METALLOTHIONEINS OF THE SOUTH AMERICAN OPOSSUM, *MONODELPHIS DOMESTICA*

by

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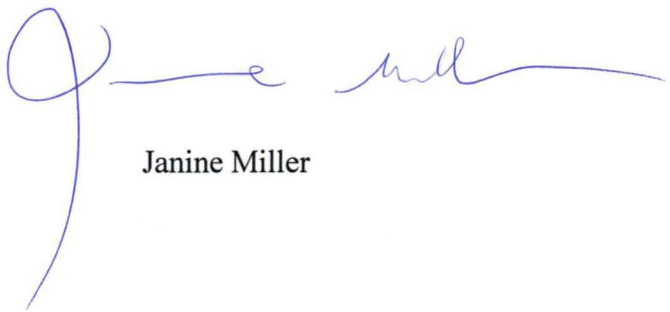
STATEMENTS

This thesis contains no material which has been accepted for a degree or diploma by the University or any institution, except by way of background information and duly acknowledged in the thesis, and to the best of my knowledge and belief no material previously published or written by another person except where due acknowledgement is made in the text of the thesis.



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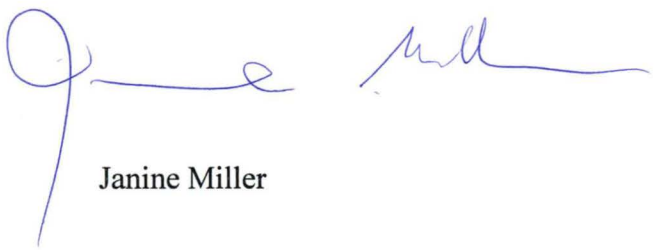

Janine Miller

ABSTRACT

Metallothioneins are small, cysteine-rich, metal-binding proteins which occur ubiquitously throughout the animal kingdom. After several decades of research, a definitive function for these metallothioneins has yet to be clarified. Many metallothioneins have been characterised in the placental mammals (subclass Eutheria) and their occurrence in complex multigene families has been confirmed. Subtle differences in protein structure and expression exist between metallothionein isoforms but it has yet to be established whether the various isoforms play differing roles physiologically. Of the vertebrates, the mammals alone manifest complex MT gene families. The birds, by contrast, express one or rarely two metallothionein genes.

The marsupials represent a mammalian subclass (Metatheria) distinct from the placental mammals. Gross differences in reproductive biology distinguish the two groups, but in other respects the marsupials differ only slightly from placental mammals in fundamental physiology. From several perspectives, characterisation of a marsupial MT should extend our understanding of the physiological role and the evolution of the mammalian metallothioneins. Marsupial metallothionein is identified here for the first time.

Several independent marsupial MT sequences were isolated from both liver and brain-derived cDNA libraries. The predicted marsupial MT proteins do not conform closely to eutherian isoforms when the traits of cognate proteins are compared, and cannot therefore be accommodated within the current classification system. The tissue-specific expression of the marsupial metallothioneins was investigated to further clarify their relationship to the eutherian metallothioneins. Here too, no confident correlation is possible. The specific and predictable expression profile of MT in the perinatal eutherian was compared with that of the marsupial neonate, which is relatively and strikingly underdeveloped. Marked differences in these profiles were observed.

Like the eutherian mammals, the marsupial mammals are here shown to express a complex family of metallothionein genes. Phylogenetic analysis of the

vertebrate metallothionein gene family indicates that the amplification of ancestral genes into multiple isoforms occurred independently in both mammalian lineages. It can be inferred that the multiplicity of MT isoforms has arisen not just in response to the physiological requirements that distinguish the mammals, but to some stimulus that acted upon their particular physiology a considerable time after its establishment. Two metallothionein cDNA sequences from the echidna, an Australian monotreme of the mammalian subclass Prototheria, are included in the analysis and confirm these conclusions. It is shown that the mammalian metallothioneins have evolved from an MT3 homologue closely related to bird MT. The eutherian MT1 and MT2 isoforms arose subsequent to the separation of the eutherian and metatherian lineages, approx. 120 myrs ago. The position of the MT4 isoform is less clear but is likely to represent the most ancient of the metallothionein proteins.

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ABBREVIATIONS

AD -	Alzheimer's disease
ANGIS -	Australian National Genome Information Service
ATP -	adenosine triphosphate
bp -	base pairs
CHO -	Chinese hamster ovary
cDNA -	complementary DNA
CNS -	central nervous system
CSF -	cerebrospinal fluid
dATP -	deoxyadenosine triphosphate
dCTP -	deoxycytodine triphosphate
DEPC -	diethyl pyrocarbonate
DNA -	deoxyribonucleic acid
DNAse -	deoxyribonuclease
dNTPs -	deoxyribonucleotides
GSH -	reduced glutathione
GSSG -	glutathione disulphide
GTP -	guanidine triphosphate
EDTA -	ethylene diamine tetra-acetic acid
icefish A -	*Chaenocephalus aceratus*
icefish B -	*Chionodraco ratrospinosus*
icefish C -	*Parachaenichthys charcoti*
indel -	insertions and deletions
IPTG -	isopropyl-β-D-thiogalactopyranose
IUPAC-IUB -	International Union of Pure and Applied Chemists - Union of Biochemists
JC -	Jukes-Cantor (transformation)
JN -	Jin & Nei (transformation)
K-2-P -	Kimura-2-parameter (transformation)
kb -	kilobase
kD -	kildaltons
LPS -	lipopolysaccharide
MdMT -	*Monodelphis* metallothionein, or the cDNA fragment described in Fig. 3.3.
ML -	maximum likelihood
mMT3 -	mouse metallothionein 3
mMT4 -	mouse metallothionein 4
MP -	maximum parsimony
mRNA -	messenger ribonucleic acid
MT -	metallothionein
NJ -	neighbour joining (method)
nr -	non-redundant (data set)
nrx -	extended non-redundant (data set)
OD -	optical density

P -	bootstrap proportion
PCR -	polymerase chain reaction
pfu -	plaque-forming unit
RNA -	ribonucleic acid
RNAse -	ribonuclease
rRNA -	ribosomal ribonucleic acid
ROS -	reactive oxygen species
RT -	reverse transcription, reverse transcriptase
SDS-	sodium dodecyl sulphate
sMT3 -	sheep MT3
SOD -	superoxide dismutase
SSC -	saline sodium citrate
Tris -	tris[hydroxymethyl]-aminomethane
ts:tv -	transition-transversion ratio
UCP -	uncoupling protein
UTR -	untranslated region
x-gal -	5-bromo-4-chloro-3-inoyl-β-D-galactopyranoside

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CHAPTER 1: INTRODUCTION

1.1 Metallothionein

1.1.1 Definition

Metallothioneins are small (~6kD), cysteine-rich, metal-binding proteins, first isolated by Margoshes and Vallee in 1957 from equine kidney. The definition of metallothionein remains in terms of the characteristics of the original sulphur-rich protein, as recommended by the First International Meeting on Metallothionein and Other Low Molecular Weight Metal-Binding Proteins (Fowler *et al.*, 1987). Metallothioneins, therefore, are "polypeptides resembling equine renal metallothionein in several of their features".

1.1.2 Occurrence

Metallothioneins occur ubiquitously through eukaryotic and prokaryotic groups (reviewed in Hamer, 1986). Class I metallothioneins are those with a peptide structure comparable to equine renal metallothionein, and specifically a strong positional conservation of cysteine residues. The vertebrate and some invertebrate MTs fall into this class. Every vertebrate species examined has been shown to express at least one class I metallothionein or its transcript. Class II metallothioneins have peptide structure and disposition of cysteine residues removed to some extent from those of equine metallothionein. Most non-vertebrate gene-encoded metallothioneins fall into this category eg. yeast MT (Winge *et al.*, 1985), invertebrate (Dallinger, 1996), prokaryote (Huckle *et al.*, 1993) and plant MT-like proteins (Hudspeth *et al.*, 1996). Class III metallothioneins incorporate those metal-binding peptides that are not genetically-encoded, including the plant phyochelatins (Rauser, 1995), and the cadystins (Hayashi *et al.*, 1991). This thesis addresses specifically and exclusively the class I metallothioneins.

1.1.3 Structure

Vertebrate metallothioneins are an extensive and highly conserved group of small metal-binding polypeptides, ranging in length from 60-68 amino acids.

They are characterised by a lack of aromatic amino acids and a high proportion of cysteine (22-33mole%) (Hunziker & Kagi, 1985). The positional conservation of cysteines is virtually absolute (Fig. 1.1). Cysteine residues invariably occur within cys-cys, cys-x-cys or cys-x-y-cys motifs within the polypeptide, where x, y represent other non-cysteine amino acids (Otvos & Armitage, 1980). Metallothionein has no disulphide bridges. Cysteine residues are present in the reduced form, coordinated to metal ions through mercaptide bonds. Metallothionein tertiary structure is maintained by metal thiolate linkage, and the metal-free apothionein molecule exists as a random coil (Kagi *et al.*, 1984). Replacement of certain cysteines perturbs the structure of the protein by diminishing the stability of the metal-thiolate bonds (Cismowski & Huang, 1991). Disturbance of the site conservation of cysteines has also been shown to diminish the copper-binding efficiency of the carboxyterminal domain (Kille *et al.*, 1992). Lysine residues are also highly conserved in juxtaposition with cysteines (Kagi *et al.*, 1984), and are postulated to contribute to charge neutralisation at the thiolate bond site (Kojima *et al.*, 1976; Vasak *et al.*, 1985; Pande *et al.*, 1985), with consequent effects on metal-binding lability (Pan *et al.*, 1994). The conserved lysines of MT also contribute to the ATP-binding site of the protein (Jiang *et al.*, 1998b).

MT is comprised of two domains (Otvos & Armitage, 1980), the aminoterminal β-domain including residues 1-29 (numbered for mouse MT1) and the carboxyterminal α-domain, residues 32-61 (Winge & Miklossy, 1982a) (Fig. 1.2). There is little physical contact between the two domains (Furey *et al.*, 1986) and each independently exhibits characteristics of the intact protein (Winge & Miklossy, 1982a; Nielson & Winge, 1985; Zelazowski *et al.*, 1984). The folding of each domain is topographically similar (Furey *et al.*, 1986) but with opposite chirality (Schultze *et al.*, 1988). The domains are joined by a flexible hinge of invariant lysine residues at sites 30-31. Replacement of either lysine with an uncharged amino acid results in a failure of MT expression (Cody & Huang, 1993). Cleavage of the protein at the hinge by subtisilin results in domain fragments capable of binding metal ions consistent with the stoichiometry of the intact protein (Nielson & Winge, 1985). Each domain exhibits specific metal-binding stoichiometries (Winge & Miklossy, 1982a),

Figure 1.1 Conservation of cysteine residues

The cysteine residues of class I metallothioneins are positionally conserved along the polypeptide, in cys-cys, cys-x-cys and cys-x-y-cys motifs (taken from Kagi *et al.*, 1984).

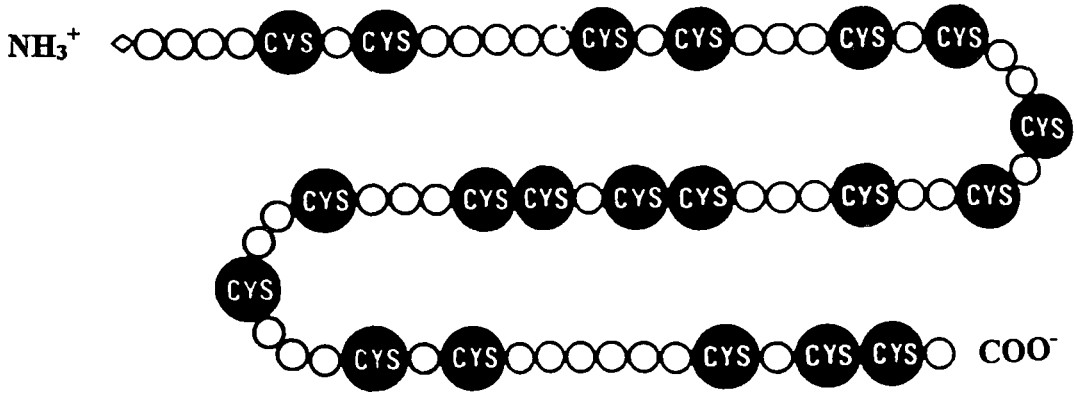
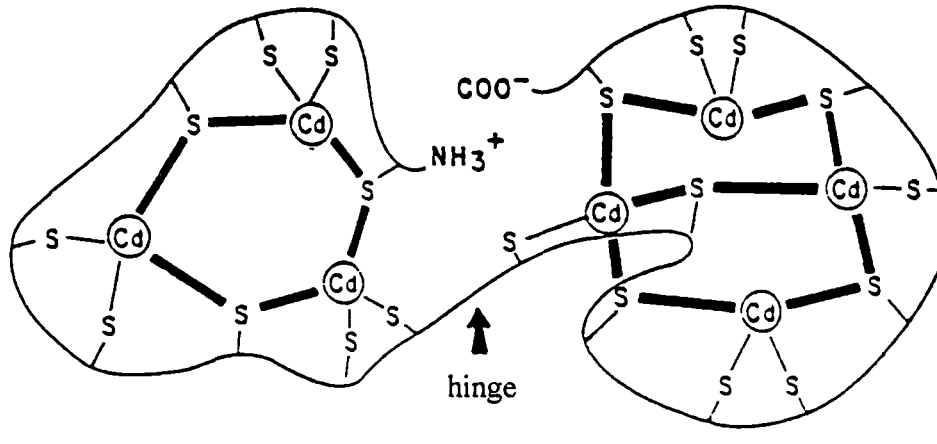


Figure 1.2 Domain structure of metallothionein

A schematic representation of the structure of Cd_7-metallothionein. The N-terminal β-domain contains three metal ions and the C-terminal α-domain contains four metal ions. Each metal ion is bonded to four sulphur molecules (tetrathiolate bonding). Each cysteine molecule is bonded to either one or two metal ions (from Winge & Miklossy, 1982).



different stabilities (Bernhard *et al.*, 1986; Cismowski & Huang, 1991; Kurasaki *et al.*, 1996) and characteristic metal proclivities (Otvos & Armitage, 1980; Briggs & Armitage, 1982; Nielson *et al.*, 1985; Stillman & Zelazowski, 1988). The structural plasticity of the metallothionein molecule allows the accommodation of metal ions of very different sizes without concomitant changes in coordination geometry or protein conformation (Kagi, 1993).

1.1.4 Metal-Binding Properties

Metallothionein binds transition Ib and IIb metal ions, principally copper, zinc and cadmium (Kagi & Vallee, 1961; Pulido *et al.*, 1966). Of these, copper has the greatest affinity for metallothionein, followed by cadmium, then zinc (Kagi &Vallee, 1960; Rupp & Weser, 1978). Metallothioneins have been shown experimentally to bind at least 18 different metal ions (Nielson *et al.*, 1985). The metal complement of the MT molecule varies with determinant factors including species (Prinz & Weser, 1975), tissue (Heilmaier *et al.*, 1987; Misra *et al.*, 1997), stage of development (Porter, 1974; Johnson & Evans, 1980) and the bioavailability of metals (Bremner, 1976). The β-domain of the protein binds three divalent metal ions through 9 cysteine residues (M_3S_9), with tetrahedral coordination. The α-domain binds four divalent metal ions through 11 cysteine residues (M_4S_{11}), also with tetrahedral coordination. Both domains bind six Cu(I) ions, in trigonal coordination. Both homometallic and heterometallic clusters occur, and site-specific metal preferences exist within the polypeptide (Chang & Huang, 1996). While thermodynamically stable, metal-thiolate bonds are kinetically labile (Vasak *et al.*, 1985; Vasak, 1986), and those of the β-domain have been shown to be considerably more labile than those of the α-domain (Nettlesheim *et al.*, 1985; Bernhard *et al.*, 1986; Otvos *et al.*, 1987, 1989; Stillman & Zelazowski, 1989). Using molecular modelling techniques, deep clefts have been demonstrated in each domain which expose the metal-thiolate clusters to solvent (Robbins *et al.*, 1991; Fowle & Stillman, 1997), facilitating intramolecular (Vasak, 1986; Otvos *et al.*, 1987; Stillman *et al.*, 1987; Stillman & Zelazowski, 1988) and intermolecular (Nettlesheim *et al.*, 1985; Otvos *et al.*, 1987) exchange of metal ions.

1.1.5 Vertebrate Metallothionein Gene Families

1.1.5.1 Lower Vertebrates

Within the vertebrates, the complement of metallothionein proteins varies across taxonomic groups. Fish species are described as having either one (eg. pike, stoneloach) (Kille *et al.*, 1991) or two isoforms (eg. trout, carp) (Price-Haughey *et al.*, 1986; Kito *et al.*, 1982). Amphibia, including several species of frog (Suzuki *et al.*, 1983b; Yamamura & Suzuki, 1983; Suzuki *et al.*, 1986) and the axolotl *Ambystoma mexicanum* (Suzuki & Ebihara, 1984; Saint-Jacques *et al.*, 1998), have a single MT isoform. *Rana catesbeiana*, alone of the amphibians examined, has a second MT isoform (Suzuki & Akitomi, 1983). No reptile metallothionein gene or protein has yet been characterised, but single hepatic MT proteins have been detected in several species, including the alligator (Bell & Lopez, 1985), water lizard (Suzuki *et al.*, 1984) and tortoise (Yamamura & Suzuki, 1984).

1.1.5.2 Birds

Within the bird species examined, a single hepatic isoform has been isolated in turkey, pheasant, chicken, duck and muscovy (Wei & Andrews, 1988; McCormick *et al.*, 1988; Lin *et al.*, 1990b; Shartzer *et al.*, 1993; Lee *et al.*, 1996). A second minor transcript was isolated from the quail, but the single nucleotide difference from the major form suggests it is probably an experimental arefact (Shartzer *et al.*, 1993). The single bird MT isoform shows a remarkable absolute conservation of the primary sequence across species, particularly striking because the divergence date of ducks (order Anseriformes) and chickens (order Galliformes) is estimated at 103 million years ago (Sibley *et al.*, 1988). Southern analysis of genomic DNA from these species indicates no further *MT* gene (Fernando & Andrews, 1989; Fernando *et al.*, 1989; Lin & Huang, 1990; Andrews & Fernando, 1991). The pigeon is the only bird species with a demonstrated and convincing second hepatic isoform (Lin *et al.*, 1990a). While the predominant isoform is largely (94%) similar in amino acid sequence to that of other birds, the second departs considerably, with only 68% sequence

similarity. Neither genomic analysis nor examination of non-hepatic tissues has been performed to establish the existence of further pigeon metallothioneins.

1.1.5.3 Mammals

The mammalian metallothinein locus has been multiply duplicated. The mouse gene family occurs as a tandem array of closely linked genes on chromosome 8 (Cox & Palmiter, 1983; Quaife *et al.*, 1994). It is the simplest of the known metallothionein gene families, coding four distinctive MT isoforms, MT1-MT4. Individual isoforms can be distinguished on several criteria. *MT1* and *MT2* occur within 6kb of one another (Searle *et al.*, 1984), and are coordinately expressed as part of a "transcription unit" (Yagle & Palmiter, 1985). *MT3* lies outside this unit, more than 20kb upstream from the *MT2* gene, with *MT4* a further 20kb upstream (Quaife *et al.*, 1994). The order Rodentia has been shown to have separated from the eutherian lineage 115 million years ago (Jancke *et al.*, 1997), very early in the eutherian radiation. Therefore, it is reasonable to presume that the relatively simple rodent *MT* gene family may represent the prototypic *MT* family of the eutherians.

The human metallothionein locus consists of 16 contiguous genes on chromosome 16q13 (West *et al.*, 1990; Palmiter *et al*, 1992; Quaife *et al.* , 1994), and represents the most complex metallothionein gene family so far described. Four distinctive isoforms correspond to those of the mouse, with further multiple duplication of the *MT1* gene. Of the thirteen *MT1* genes, eight are transcriptionally active: *MT1a* (Richards *et al.*, 1984), *MT1b* (Heguy *et al.*, 1986), *MT1e* (Schmidt *et al.*, 1985), *MT1f* (Schmidt *et al.*, 1985; Varshney *et al.*, 1986), *MT1g* (Foster *et al.*, 1988), *MT1l* (Holloway *et al.*, 1997b), *MT1h* and *MT1x* (Stennard *et al.*, 1994). Transcription of the remaining five *MT1* genes has not been demonstrated, and various aberrations in nucleotide sequence suggests that these genes do not produce a complete transcript: *MT1c* and *MT1d* (Richards *et al.*, 1984), *MT1i*, *MT1j* and *MT1k* (Stennard *et al.*, 1994). *MT2* lies upstream from and within 7-8kb of the *MT1* genes (West *et al.*, 1990). The sheep metallothionein locus has been estimated to comprise at least ten members (Peterson *et al.*, 1988; Holloway, 1996), and the partial definition of the family (Peterson *et al.*, 1984, 1988) suggests that the expanded locus is also *MT1*.

Metallothioneins of the rabbit have been isolated principally as proteins (Hunziker *et al.*, 1995). Tissues of Cd-treated rabbits and cultured kidney cells express at least seven separable metallothioneins, one of which conforms to an MT1 and the remaining 5-6 to MT2. (It is unclear whether MT2b and MT2b' are coded by separate genes). Therefore, the rabbit represents an instance in which the *MT2* gene rather than the *MT1* gene has been multiply duplicated.

The four major isoforms of the eutherian MT family have yet to be demonstrated in species other than the mouse and human. However, the presence of *MT3* and *MT4* genes in both these genomes implies their existence in the lineage before the eutherian radiation, and justifies the expectation that both these isoforms will occur throughout the eutherian mammals. The MT1 and MT2 isoforms have been demonstrated in many mammalian species, and multiple duplication of one or other gene occurs in all species closely examined, with the exception of the rodents.

1.1.6 MT isoforms of eutherian mammals

The four characteristic isoforms of the eutherian mammals can be distinguished on several criteria.

1.1.6.1 MT1 and MT2

MT1 and MT2 isoforms were originally defined as charge separable hepatic proteins. However, the distinction has been modified to signify the presence (MT2) or otherwise (MT1) of an acidic residue at either site 10 or 11 of the polypeptide chain (Kagi & Kojima, 1987). All mammals examined to date express both isoforms. This expression is very widespread throughout the organism, and is most conspicuous in the parenchymatous and epithelial tissues (Durnam & Palmiter, 1981; Danielson et al., 1982a). The two mouse isoforms are coordinately regulated through a chromosomal domain (Yagle & Palmiter, 1995), and their expression is correspondingly similar. The broad tissue distribution of both isoforms is comparable in other mammals in which regulation is not coordinate, though the level of expression can vary between isoforms (eg. Peterson & Mercer, 1988).

The expression of metallothioneins 1 and 2 is inducible by many factors, including metals, glucocorticoids and cytokines (reviewed in Kagi, 1993). As aforementioned, one or other of the isoforms is encoded by multiple genes in many mammals studied to date, and variation exists in the occurrence of the different subforms in both the level of expression, inducibility and tissue-type-specificity. For instance, *MT2* and *MT1x* are transcribed in four human breast cancer cell lines, but *MT1a*, *MT1b*, *MT1f*, *MT1g*, and *MT1h* are not. Varying levels of *MT1e* are transcribed in the different cell lines and these levels correlate with concentrations of estrogen receptor in the cell (Friedline *et al.*, 1998). In the NIH3T3 human cell line, *MT1a* has been shown to be maximally induced by cadmium, whereas *MT2* is maximally induced by zinc and dexamethasone (Richards *et al.*, 1984). Furthermore, the MT1 content of prenatal eutherian liver is higher than that of MT2 (Suzuki *et al.*, 1983a; Knudsen & Beattie, 1997).

The characteristics of the two hepatic MT isoforms differ in many ways. For instance, the MT1 protein displays a shorter half-life for than MT2 under various induction regimes (Kershaw & Klaassen, 1992). The free radical scavenging capacity of both isoforms has been shown to differ (Kumari *et al.*, 1998), and factors relating to the metal composition of each can also vary (Lehmann-McKeeman *et al.*, 1988a, 1988b). For example, MT2 has a higher affinity for zinc than does MT1 (Winge & Miklossy, 1982b).

Isoform-specific antibodies directed against MT1 and MT2 are not commercially available. The commonly used monoclonal antibodies (eg. E9) recognise an epitope at the first seven residues of the polypeptide (Garvey,1984) that is invariant in the MT1 and MT2 isoforms. Therefore, metallothioneins 1 and 2 can be detected immunologically but not distinguished. The epitope is disrupted in MT3 and MT4, and consequently these isoforms are not considered to cross-react with these antibodies. However, Tohyama *et al.* (1996) report residual levels of antigenicity to MT1/2-directed antibody in the testis, brain and tongue of *MT*-null mice. Their conjecture that this corresponds to some cross-reactivity of other isoforms with their antibody is supported by the subsequent demonstration (Moffatt & Seguin, 1998) of *MT3* transcripts in all three tissues.

1.6.1.2 MT3

MT3 was isolated originally as a neural growth inhibitory factor down-regulated in the Alzheimer's brain (Uchida *et al.*, 1991). Initially considered a brain-specific member of the mammalian gene family (Tsuji *et al.*, 1992), *MT3* has subsequently been shown to be expressed in the pancreas and intestine, albeit at low levels (Erickson *et al.*, 1995), in human renal proximal tubules and cultured kidney cells (Hoey *et al.*, 1997), and most recently in male and female reproductive organs, tongue, heart and stomach of the rat (Moffatt & Seguin, 1998). Evidence for the transcription of *MT3* and *MT4* genes in the maternal deciduum of the mouse, together with *MT1* and *MT2*, constituted the first documented instance of coordinated transcription of the complete *MT* locus in a single tissue or cell type (Liang *et al.*, 1996). The MT3 isoform is distinguished by sequence insertions in both domains: a single glutamic acid* insertion after residue 3 (numbering as mouse MT1) and a 4-6 residue insertion between residues 52 and 53. The sequence of the latter insertion is not conserved between species. All known MT3 amino acid sequences contain a Cys-Pro-Cys-Pro motif in the β-domain, to which is attributed the growth inhibitory activity of the human isoform (Sewell *et al.*, 1995). MT3 is not subject to the same regulation as isoforms MT1/2 and is not susceptible to induction by metals, glucocorticoids, or other known inducers of MTs1 and 2 (Palmiter *et al.*, 1992; Masters *et al.*, 1992b). Antibodies specific to MT3 have been reported (Uchida *et al.*, 1991; Tohyama *et al.*, 1996; Yamada *et al.*, 1996), but are not easily prepared or obtained. Therefore, much analysis of *MT3* expression is based on the detection of transcript (Masters *et al.*, 1994b; Choudhuri *et al.*, 1995).

* The site of N-terminal insertion in both MT3 and MT4 occurs between invariant Pro3 and Cys5. In this work, the format of Kagi (1993) is followed, with insertions deemed to occur after residue three.

1.6.1.3 MT4

Existence of the *MT4* gene was established by analysis of sequences upstream from the metallothionein loci of both mouse and human genomes (Quaife *et al.*, 1994). The protein has all the structural characteristics that define

a class I metallothionein, including positionally invariant cysteine residues. A single glycine * insertion occurs after residue 3 in the N-terminal domain of the protein, relative to mouse MT1. The most striking feature of the MT4 isoform is its extremely localised distribution. *In situ* hybridisation demonstrates the occurrence of *MT4* transcript in a specific cell layer, the *stratum spinosum*, of the stratified squamous epithelia of cornified tissues in the mouse, specifically on the surfaces of the tongue, footpad, upper oesophagus and tail. *MT4* expression is thought to co-localise with the differentiating stage of cells which synthesise a particular form of keratin. Recent studies have demonstrated the coordinated expression of the complete metallothionein locus in the mouse maternal deciduum during early gestation (Liang *et al.*, 1996). No antibody to MT4 has been reported.

1.1.7 Some Proposed Physiological Functions

1.1.7.1 Metal detoxification

Metallothionein has frequently been implicated in the detoxification of heavy metals, presumably by sequestration, and a literary corpus exists on the metal detoxifying properties of MT (eg. Leber & Miya, 1976; Bremner & Beattie, 1990). The expression of metallothionein is induced in many tissues (Nordberg *et al.*, 1971) and cultured cell lines (Karin *et al.*, 1981) by metals. The inducing metals are usually sequestered by apothionein molecules post-translationally, though MT does not detoxify all metals by which it is induced (Durnam & Palmiter, 1984). Enhanced expression of MT in cultured cell lines through transgenesis (Karin *et al.*, 1983; Schmidt *et al.*, 1985) or amplification (Beach & Palmiter, 1981; Mayo & Palmiter, 1982; Crawford *et al.*, 1985) demonstrably increases resistance to the effects of metal toxicity. Conversely, cells in which metallothionein expression is deficient due to DNA methylation (Compere & Palmiter, 1981) or mutagenesis (Masters *et al.*, 1994a) are more sensitive to the toxic effects of cadmium than are control cells. Similarly, transgenic mice which over- (Liu *et al.*, 1995) or under-express (Michalska & Choo, 1993; Masters *et al.*, 1994a) metallothionein display corresponding increases in resistance and susceptibility, respectively, to the detrimental effects

of heavy metals. Interestingly, NIH3T3 cells transfected with the mouse *MT1* gene display a 10-fold resistance to cadmium, a significant proportion of which is attributed to reduced uptake of metal into the cell (Morton *et al*, 1992).

The cytoprotective role of metallothioneins in relation to heavy metals is clear. However, it is less clear that this is the primary physiological role of the protein (Karin, 1985; Webb, 1987). MT is a zinc-binding protein. The chemical similarity of cadmium and zinc allows MT a fortuitous function in the sequestration of the former, and a role for MT in physiological detoxification of cadmium may therefore be adventitious. Furthermore, the redox properties attributed to MT that mediate zinc availability in the cell (Maret & Vallee, 1998) would compromise the stability of MT as a reservoir for toxic metals. These roles seem mutually exclusive.

1.1.7.2 Metal Homeostasis

Zinc and copper, the most commonly bound metals of MT, are elements essential for the normal metabolic processes of the cell. Both metals are necessary constituents of many enzymes, cofactors and cellular components, including an extensive array of transcription factors, polymerases and synthases. Zinc also acts in cellular processes, including neuromodulation (Assaf & Chung, 1984; Peters *et al.*, 1987). Little free zinc is found in the cell, but is almost invariably complexed with proteins. MT is unique in binding zinc and copper with a high thermodynamic stability, but also with a strong kinetic lability (Vasak *et al.*, 1985; Vasak, 1986). The role of MT in the storage, release and exchange of cellular zinc has recently received considerable attention.

Zinc is a redox-inert metal. It has been proposed that the cysteine ligands of MT confer upon bound zinc a redox potential that allows the availability of MT-bound zinc to be controlled by the intracellular redox status. Because this redox status is determined by physiological conditions, the reactivity of MT-bound zinc becomes responsive to the physiological state of the cell (Maret, 1995). Cellular disulphides such as GSSG, coenzyme A disulphide and cystameine have been shown to initiate S-thiolation of the cysteine-metal bond of MT, facilitating the release of metal ions from the protein *in vitro*, at physiological concentrations (Maret, 1994). This suggests that the release of

metals from MT may be a function of the GSSG:GSH redox status of the cell (or an equivalent), itself the product of various physiological conditions (Gilbert, 1990). Oxidative stress is one such arbiter of intracellular redox status. Cd_5Zn_2MT has been shown to form complexes with GSH (Brouwer *et al.*, 1993). Reduced glutathione may, therefore, protect MT from metal-release by shielding thiolate sites when the GSSG:GSH ratio is low. Other redox couples demonstrated to initiate metal-release from MT *in vitro* include H_2O_2/H_2O, HNO_2/NO, ferricyanide/ferrocyanide, cytochrome c (FeIII/FeII) and selenite/selenium, though disulphides and selenite are considered the most likely effectors *in vivo* (Maret & Vallee, 1998).

Transfer of zinc ions from donor metallothionein has been documented for several (apo)enzymes: carbonic anhydrase (Li *et al.*, 1980; Udom & Brady, 1980; Kraker *et al.*, 1988), alcohol dehydrogenase, aldolase and thermolysin (Udom & Brady, 1980) and sorbitol dehydrogenase (Jiang *et al.*, 1998a). Zinc transfer to an acceptor thionein molecule from zinc-containing enzymes has also been noted for transcription factors IIIa and Sp1 (Zeng *et al.*, 1991a and 1991b, respectively). Reciprocal exchange has been demonstrated for the oestrogen-receptor (Cano-Gauci & Sarkar, 1996), carboxypeptidase and alkaline phosphatase, though removal of zinc from the latter could only be achieved in the presence of other additional chelating agents (Jacob *et al.*, 1998). Reciprocal metal exchange predicts a regulatory role for MT in the activity of zinc-containing enzymes. Restoration of the activity of metal-depleted aldolase, thermolysin (Udom & Brady, 1980), pyroxidal kinase (Churchich *et al.*, 1989) and sorbitol dehydrogenase (Jiang *et al.*, 1998a) by metal transfer from MT has been demonstrated *in vitro*. GSSG and GSH both participate in regulating the exchange of zinc between MT and sorbitol dehydrogenase. It is considered unlikely that zinc transfer between MT and apoenzymes occurs *in vivo* without the intervention of the GSSG/GSH couple (Jiang *et al.*, 1998a), because the inhibition of metal release by GSH prevails at physiological steady state (Maret & Vallee, 1998). Metal transfer reactions from MT to apoenzymes are not restricted to MT-bound zinc. Reciprocal transfer of copper between CuMT and β-monooxygenase has been reported (Markossian *et al.*, 1988), and from

metallothionein to ceruloplasmin (Schechinger *et al.*, 1986) and Cu,ZnSOD (Suzuki & Kuroda, 1995).

A complex of ATP or GTP with metallothionein has recently been demonstrated to ehance the transfer of zinc to zinc-depleted sorbitol dehydrogenase (Jiang *et al.*, 1998b). The formation of this complex is also dependent on the GSSG:GSH status: at low GSH, MT may be ATP-saturated. A combination of cellular redox potential and energy state, therefore, would seem to dictate the activity of MT in intracellular metal storage, release and transfer, with concomitant regulation of enzyme function. Maret & Vallee (1998) propose that the primary role of MT is in the mediation of cellular zinc distribution as a function of the energy state of the cell. In this way, MT acts as an inert pool of reactive zinc within the cell, with the capacity for controlled and rapid metal release as necessary.

The metal storage and dispensation role of MT may account for the high levels of MT observed during early development (Bakka & Webb, 1981; Brady *et al.*, 1982) and in proliferating tissue (eg. Tohyama *et al.*, 1993), when synthetic processes are maximal and the consequent metabolic requirement for metals is high. The redox control of MT reactivity adds another level to the already complex regulation of cellular metallothionein.

1.1.7.3 Free Radical Scavenger

Both normal and pathological processes generate reactive oxygen species (ROS). The potential destructiveness of these molecules to lipid membranes, DNA and cellular proteins is countered by cellular antioxidant molecules including catalase, superoxide dismutase and peroxidase. Data is accumulating to justify the inclusion of metallothionein among the cellular antioxidants. MT has demonstrable hydroxyl radical scavenging capacity *in vitro* (Thornalley & Vasak, 1985; Abel & de Ruiter, 1989), attributed to its particularly high sulphydryl content, thought to constitute the sites of hydroxyl radical interaction (Thornalley & Vasak, 1985; Quesada *et al.*, 1996; Jimenez *et al.*, 1997).

Hydroxyl radicals are generated markedly in cells exposed to high energy radiation. Whole body X-irradiation has been shown to induce *MT1* in an exposure-dependent manner in mice (Koropatnick *et al.*, 1989), while pre-

exposure of mice to zinc ameliorates the detrimental effects of irradiation (Matsubara, 1987). Induction of MT with either heavy metals or cytokines in cultured mammalian cell lines has been correlated with an increased resistance to oxidative stress generated by hydrogen peroxide (Mello-Filho *et al.*, 1988), organic peroxides (Ochi *et al.*, 1988), hyperoxia (Hart *et al.*, 1990) and carbon tetrachloride (Schroeder & Cousins, 1990). However, these inducing agents affect other cellular factors, including the antioxidant glutathione. When pre-treatment with cadmium protected cells against the effects of oxidative stress, glutathione was shown to be the induced and mediating factor, rather than metallothionein (Chubatsu *et al.*, 1992). Furthermore, the over-expression of *MT* in Chinese hamster ovary (CHO) cells after gene transfection failed to confer resistance to ionizing radiation or to alkylating agents (Lohrer & Robson, 1989). Transfection of NIH3T3 cells with mouse *MT1*, on the other hand, increased the antioxidant activity of these cells, demonstrated by an increased resistance to the effects of the prooxidant *tert*-butyl hydroperoxide (tBH) (Schwarz *et al.*, 1994). Cultured embryonic cells from *MT1/2*-null mice display enhance sensitivity to tBH-induced oxidative stress (Lazo *et al.*, 1995).

It has been suggested that the role of MT is not to counter the lethal effects of ROS on cells but to confer protection on specific cellular components against oxygen-mediated injury (Chubatsu & Meneghini, 1993). Oxidation of MT has been shown to cause structural perturbation of the molecule, with possible novel properties arising (Fang *et al.*, 1994). Recent studies have shown that MT partially oxidised by radiation-induced hydroxyl radicals will bind DNA in proportion to the degree of oxidation (Fang *et al.*, 1997). These data support earlier findings that cells over-expressing MT display less H_2O_2-generated DNA strand scission than control cells (Martins *et al.*, 1991; Chubatsu & Meneghini, 1993). The occurrence of MT in the cell nucleus under certain circumstances (Panemangalore *et al.*, 1983; Nartey *et al.*, 1987; Nishimura *et al.*, 1989; Tohyama *et al.*, 1993) supports a role for MT in the protection of DNA from hydroxyl-mediated damage. The subcellular location of MT is deemed important for the site of its protective action within the cell (Schwarz *et al.*, 1994). Evidence also exists that links MT with the inhibition of hydroxy-induced lipid peroxidation (Markant & Pallauf, 1996).

Although MT has a relatively low reactivity with superoxide radicals (Thornalley & Vasak, 1985), mammalian and yeast MT have been shown to effectively rescue copper/zinc superoxide dismutase (Cu,ZnSOD) mutants in yeast (Tamai *et al.*, 1993). It is unclear, however, whether it is the SOD-dismutase or the copper-buffering activity (Culotta *et al.*, 1995) of Cu,ZnSOD that is substituted by metallothionein.

1.1.7.4 Growth Inhibitory Function

MT3 was first isolated as a growth inhibitory factor deficient in the Alzheimer's diseased (AD) brain (Uchida *et al.*, 1991). Although the down-regulation of *MT3* in the AD brain has subsequently been challenged as statistically insignificant (Erickson *et al.*, 1994), the inhibitory activity of the isoform is demonstrable *in vitro*. In the presence of normal human or AD brain extract, MT3 inhibits the growth of cultured rat neurons, whereas MT1 does not. In the absence of brain extract, both MT1 and MT3 are neurotrophic (Erickson *et al.*, 1994). Because this latter attribute as been demonstrated for β-mercaptoethanol, dithiothreitol and glutathione as well as the MTs, it is ascribed to the antioxidant activity of these molecules (Sewell *et al.*, 1995). MT3 is, therefore, not directly toxic to neurons but may directly or indirectly inactivate a neurotrophic factor in brain extract, or conversely, activate an inhibitory factor (Erickson *et al.*, 1994). The growth inhibitory activity of MT3 is localised to the CPCP motif in the N-terminal domain of the protein (Sewell *et al.*, 1995), which is uniquely and invariably conserved in all MT3 proteins so far characterised (eg. Palmiter *et al.*, 1992; Tsuji *et al.*, 1992; Kobayashi *et al.*, 1993; Chen *et al.*, 1996; Holloway, 1996). Only pigeon MT1 has a partially similar motif, CPC, at a corresponding site (Lin *et al.*, 1990a). The physiological relevance of the growth inhibitory action of MT3 has not been firmly established *in vivo*.

When MT3 transcript in two transformed glial cell lines was assessed with quantitative PCR, levels of mRNA were found to be markedly reduced relative to those of cultured rat astrocytes (Amoureux *et al.*, 1995b). Solution hybridisation was ineffective in detecting any MT3 transcript in transformed cell lines (Masters *et al.*, 1994b). This correlates with the sustained proliferative activity of the transformed cells. When these transformed cell lines are

transfected with 5-$HT_{1D\alpha}$, resulting in a decrease in basal proliferation, MT3 mRNA exhibits a 30-fold increase (Amoureux *et al.*, 1995b). The relationship between 5-$HT_{1D\alpha}$, MT3 and the attenuation of cell growth is unclear. However, these phenomena relate an increase in MT3 expression with a down-regulation of cell proliferation, and are, therefore, consistent with a growth inhibitory action for this isoform.

MT3 was stably tranfected into CHO-K1 cells, which do not express endogenous MT, to evaluate its effects on cell growth. Growth in the presence of serum was indistinguishable between transfected and control cells. However, in serum-free medium, transfected cells have a survival rate 40% higher than controls (Amoureux *et al.*, 1995a). When baby hamster kidney (BHK) cells were transformed with either *MT1* or *MT3* genes (Palmiter, 1995), both cell lines exhibited elevated resistance to heavy metals. However, MT3 was shown to donate less zinc to zinc-requiring reporter gene products than MT1. Furthermore, *MT3*-transfected cells displayed growth retardation relative to *MT1*-transfected cells in zinc deficient medium. It is postulated that the sequestration of zinc by MT3 effectively removes it from cellular processes, instigating an inhibition of growth when zinc is limited, whereas the MT1 isoform readily donates its constitutent zinc to maintain function in zinc-requiring proteins of the cell. These data emphasise that the growth inhibitory and trophic roles of MT3 are dependent on physiological context.

Transgenic mice constructed to over-express human *MT3* display a tissue- and cell-specific profile comparable with that of control mice. Zinc levels in the brain increase in these mice, and this zinc is fully accounted for by sequestration in the excess MT (Erickson *et al.*, 1995). No neuronal or developmental dysfunction, which might be predicted with the over-expression of a growth inhibitory factor, is evident. *MT3*-null mice show no quantifiable deleterious effects, either physiological or behavioural, apart from an increased susceptibility to the effects of kainic acid-induced seizure, which implicates MT3 in a zinc management role rather than a growth inhibitory function (Erickson *et al.*, 1997). Transgenic mice that express MT3 under an *MT1* promoter, and therefore in a wide range of tissues, frequently do not survive past 2-3 months. Death is attributed to degeneration of the acinar cells of the

pancreas, though other organs show no gross or histological irregularities (Quaife *et al.*, 1998). The significance of these findings, especially with regard to the growth inhibitory activity of MT3, is not clear.

1.1.7.5 MT-Null Mice

The role of metallothionein has been investigated using transgenic mice in which the *MT1* and *MT2* genes are disrupted (*MT1*$^{-}$*/MT2*$^{-}$). These knockout mice show few physiological abnormalities when compared to control mice (Michalska & Choo, 1993; Masters *et al.*, 1994a), apart from a moderate obesity (Beattie *et al.*, 1998). They are reproductively normal (Michalska & Choo, 1993; Masters *et al.*, 1994a). An heightened sensitivity to cadmium (eg. Michalska & Choo, 1993; Masters *et al.*, 1994a; Klaassen & Liu, 1998) and an increased reaction to both zinc depletion and zinc toxicity (Kelly *et al.*, 1996), as well as greater susceptibility to UVB irradiation injury (Hanada *et al.*, 1998), suggest a protective function for MT under circumstances of environmental stress. Cell cultures derived from these strains are demonstrably hypersensitive to both oxidative stress (Lazo *et al.*, 1995) and anticancer drugs (Kondo *et al.*, 1995). Because *MT1/2*-null mice are effectively asymptomatic under normal conditions, it has been suggested that metallothioneins fulfil a redundant role in the organism or cell, as a backup or surrogate mechanism for other cellular systems, especially under conditions of physiological stress (Palmiter, 1998). When mutant mice lacking the gene for copper effluxing ATPase (strain *Mo-brJ*) were crossed with metallothionein 1/2-null mice, mortality of the progeny was accelerated relative to the *Mo-brJ* strain (Kelly & Palmiter, 1996), supporting the proposition that MT1 and MT2 provide a second line of defense against copper toxicity.

MT3^{-}*/MT3*$^{-}$ mice, which express no MT3, are also grossly asymptomatic (Erickson *et al.*, 1997). No effect has been observed on behaviour, cerebral zinc regulation or aging. Unlike the *MT1/MT2*-null mouse, the *MT3*-null mouse is no more sensitive to systemic metal toxicity than is its normal counterpart. Only a susceptibility to the effects of kainic acid-induced siezures characterises the MT3 knock-out mouse. Kainic acid-induced siezure results in greater damage to

neurons in specific regions of the hippocampus than is seen in normal control mice, possibly implicating MT3 in the regulation of hippocampal zinc.

1.1.8 Somatic Amplification of the *MT* Locus

Amplification of the MT locus has been demonstrated in cultured cells from several sources: yeast (Tohyama *et al.*, 1996), mouse (Beach & Palmiter, 1981), hamster (Hayashi *et al.*, 1983a, 1983b; Stallings *et al.*, 1984), rabbit (Wan *et al.*, 1995) and human hepatoma cells (Czaja *et al.*, 1991). Amplified copy number can range from 2-75 in CHO cells (Gick *et al.*, 1982), and up to 30-fold in yeast (Mehra *et al.*, 1990). Amplification has been shown to include both *MT1* and *MT2* loci in human (Czaja *et al.*, 1991), mouse (Thibodeau *et al.*, 1992) and hamster cells (Stallings *et al.*, 1984; Griffith, 1985; Crawford *et al.*, 1985; Yamada *et al.*, 1994). In rabbit kidney cells, highly metal-resistant cells displayed a three-fold amplification of two genes, while less resistent cells a two-fold amplification of one gene (Wan *et al.*, 1995), suggesting the level of selection may dictate both copy number and the number of genes amplified. Since the recognition of four isoforms at the mouse *MT* locus, it has been demonstrated that selection for cadmium resistance in hepa-1A cells results in amplification of the whole locus, though the *MT3* and *MT4* genes are transcriptionally silent (Quaife *et al.*, 1994). In the yeast *Candida glabrata*, the *MT2* gene is tandemly amplified 30-fold, while *MT1* remains single copy (Mehra *et al.*, 1990). Unlike mammalian MTs, these genes are not colinear but occur on different chromsomes.

The transcriptional competence and inducibility of mouse *MT* genes is usually maintained with amplification (Koropatnick, 1988; Thibodeau *et al.*, 1992). However, glucocorticoid regulation has been lost in some instances of mouse *MT* amplification, despite preservation of 18kb of 5' flanking sequence and the maintenance of metal inducibility (Mayo & Palmiter, 1982).

This form of amplification is also demonstrable *in vivo*. Mice treated with non-lethal doses of cadmium show a 2-3 fold amplification of the hepatic *MT1* gene within 6 hours of treatment, which persists up to 3 weeks after the withdrawal of stimulus (Koropatnick *et al.*, 1985).

Amplification sometimes occurs as multiple duplication of the locus into a tandem array which can appreciably increase the length of the chromosome (Alajem *et al.*, 1988; Mehra *et al.*, 1990). Extra gene copies may also be carried on double minute chromosomes (Durnam & Palmiter, 1984), or on very small chromosomes (Beach & Palmiter, 1981). Amplification is often accompanied by tetraploidy (Hayashi *et al.*, 1983b), though increased gene copy number may be far in excess of that accounted for by genomic doubling (Beach & Palmiter, 1981).

The length of the amplification unit can vary greatly. Duplication of the whole chromosome may occur (Mehra *et al.*, 1990), though amplification can also be gene-specific (Gupta *et al.*, 1992). Amplicons of 1.25kb (Mehra *et al.*, 1990) and 2kb (Tohyama *et al.*, 1996) have been reported in yeast, which occur with or without additional chromosomal duplication. The stability of amplified genes differs. Mouse cells with amplification-based cadmium resistance lose their resistance and double minute chromosomes after four months of unselected culture (Mayo & Palmiter, 1982; Thibodeau *et al.*, 1992). Amplified *MT* genes of copper-resistant human hepatoma cells are stable for over a year without selection (Czaja *et al.*, 1991).

Tumour promoters such as 12-O-tetradecanoylphorbol-13-acetate (TPA) and aplysiatoxin have been shown to increase the frequency of MT amplification (Hayashi *et al.*, 1983a, 1983b; Herschman, 1985), as they do for other amplification-prone loci such as dihydrofolate reductase and multi-drug resistance genes (Sharma & Schimke, 1994). There is also evidence of a role for chromosomal rearrangement in gene amplification in general (Coquelle *et al.*, 1998), and for the *MT* locus in particular (Stallings *et al*, 1984; Griffith, 1985; Gupta *et al.*, 1992). Amplified sequences are often found close to the chromosomal breakpoint (Stallings *et al.*, 1984; Griffith, 1985), and fragile sites have been shown to initiate intrachromosomal amplification (Coquelle *et al.*, 1998). Rearrangement, however, is not detected in every instance of *MT* gene amplification (Thibodeau *et al.*, 1992).

Two scenarios may be postulated to describe the amplification of metallothionein genes in response to selective pressure. Amplification may occur *de novo* in all, or some, cells of a tissue or culture. Alternatively, a subset of

existing cells may carry extra *MT* genes and these cells persist under competitive advantage in the face of selection (Koropatnick, 1988).

1.1.9 Evolution of the Vertebrate Metallothioneins

The high degree of conservation between metallothioneins of vastly divergent groups of organisms leaves no doubt as to the common origin of their encoding genes (Kagi *et al.*, 1984). The vertebrate MTs are two-domain proteins: the aminoterminal domain is encoded by two exons, and the carboxyterminal domain by a single exon. The metallothionein of the mould, *Neurospora crassa*, is a single domain protein containing 7 cysteine residues and binding three metal ions (Armitage *et al.*, 1987). This conformation has been proposed as the primitive eukaryotic metallothionein gene, corresponding to exon 2 of the vertebrate *MT* (Hunt *et al.*, 1984), and containing the central "core" sequence of 10 residues conserved throughout the MTs (Nemer *et al.*, 1985). It is proposed that increased requirement for metal-binding prompted a duplication and subsequent extension of exon 2, giving rise to exon 3. Exon 2 and exon 3 together constituted the ancestral form from which arose the invertebrate gene, containing 18 cysteine residues. The further addition of exon 1 generated the gene for vertebrate MT, which contains 20 metal-binding cysteines (Hunt *et al.*, 1984; Nemer *et al.*, 1985) (Fig. 1.3).

The domain structure of the sea urchin (*Strongylocentrus purpuratus*) metallothionein is inverted with respect to the vertebrate metallothioneins (Nemer *et al.*, 1985; Harlow *et al.*, 1989), with the M_4S_{11} domain at the N-terminal part of the molecule and the M_3S_9 domain in the C-terminal half of the protein (Nemer *et al.*, 1985; Wang *et al.*, 1995). Structurally, therefore, the sea urchin and vertebrate MTs are virtually identical, and their properties consequently similar (Wang *et al.*, 1994, 1995, 1996). However, the domain structure, and therefore the corresponding exon arangement, of each is inverted with respect to the other. This degree of convergent evolution argues for a very strong selection at the structure/function interface. It is interesting to note that the class I metallothioneins, that correspond to the fundamental "vertebrate" structure, also include some of the invertebrate MTs eg. those of the sea urchin and the clam, *Mytilus edulis* (Mackay *et al.*, 1993).

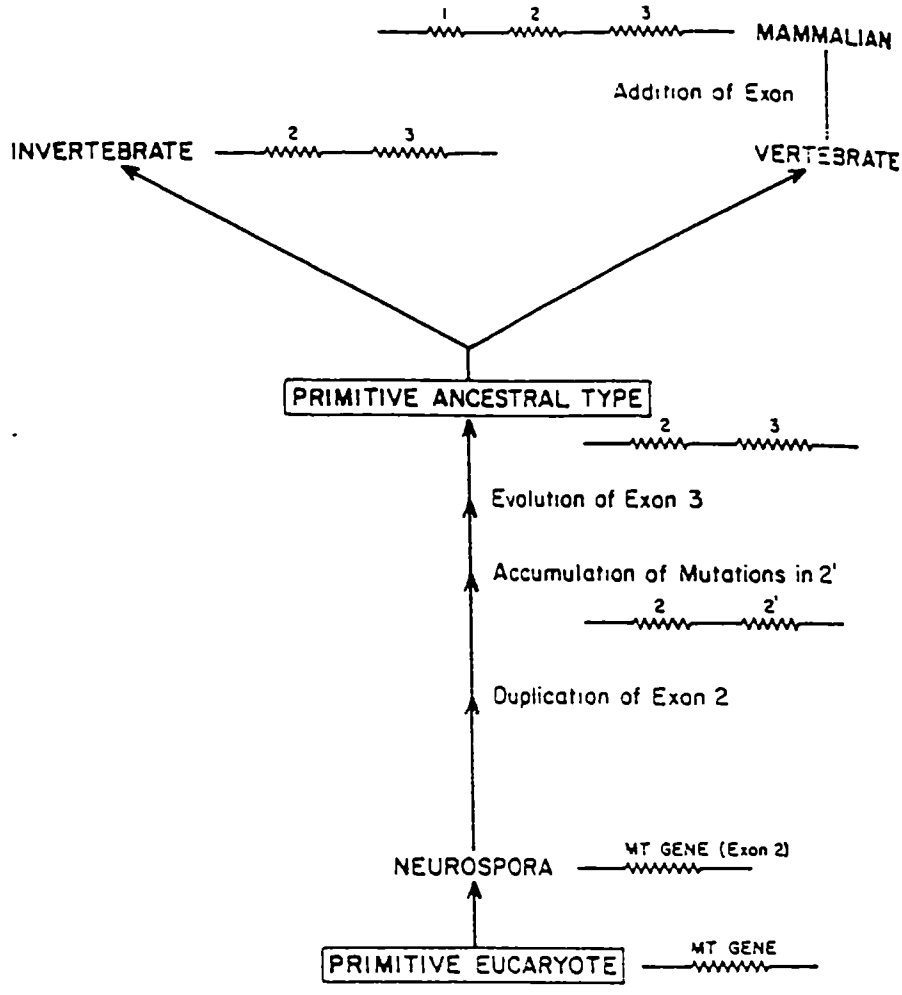


Figure 1.3 Evolution of vertebrate metallothionein

The three-exon structure of the vertebrate metallothionein gene, which gives rise to the two domains of the MT protein, is proposed to have arisen from a duplication of the single exon exemplified by the yeast *Neurospora crassa MT* gene. Divergence of the two exons produced the "primitive ancestral" form of the metallothionein gene, typical of the invertebrate *MT*s. A third exon was subsequently added to the progenitor of the vertebrate *MT* gene. It is this gene that expresses the two domain proteins known as class I metallothioneins (from Hunt *et al.*, 1984).

Using a comparison between the metallothioneins of crab (*Scylla* sp.) and mammals, Kagi *et al.* (1984) have estimated an evolutionary rate of 7.2×10^{-10} amino acid substitutions per year. This defines metallothionein as a slowly evolving protein, changing more rapidly than cytochrome c but less rapidly than haemoglobin α.

Phylogenetic analysis of the class I metallothionein proteins (Kagi, 1993) groups them largely in accord with the species phylogeny (Fig. 1.4). Invertebrate MTs are most closely related to those of fish. The mammalian MTs 1 and 2 form a coherent and exclusive cluster, which suggests a duplication of the progenitor gene that preceded the divergence of the mammalian orders. The duplication of this progenitor gene has been dated between 45 and 120 million years ago (Griffith *et al.*, 1983). The mammalian MT3 and MT4 isoforms, on the other hand, occur on the unrooted evolutionary tree (Kagi, 1993) in close association with the metallothioneins of birds and fishes, respectively. This would imply that these isoforms are more ancient than MT1 and MT2.

1.2 Mammalian Evolution

The divergence of the diapsid and therapsid reptiles, antecedents of the birds and mammals respectively, occurred 310 million years ago (Hedges *et al.*, 1996). The earliest fossil mammals are known from the Triassic, 180 million years ago (Crompton, 1980). Traditionally, it has been assumed that the Prototheria (the monotremes) evolved from one lineage deriving from the early mammals, the Multituberculata. The Metatheria (marsupials) and Eutheria (placental mammals) evolved considerably later in the Cretaceous from a second lineage, the Pantotheria (Lillegraven, 1975; Marshall, 1979). This hypothesis is known as the "Theria hypothesis" (Fig. 1.5a) and is based largely on the phenotypic similarities shared exclusively by the therians. An alternative "Marsupionta hypothesis" assigns the monotremes and marsupials to a single lineage, the Marsupionta, evolving independently of the placental mammals (Fig. 1.5b). The phenotypic characters used to justify both hypotheses have been refuted on both sides as inappropriate bases for phylogenetic inference.

When the question is addressed from a molecular perspective, varying support for each hypothesis ensues. Analyses of myoglobin genes (Goodman *et*

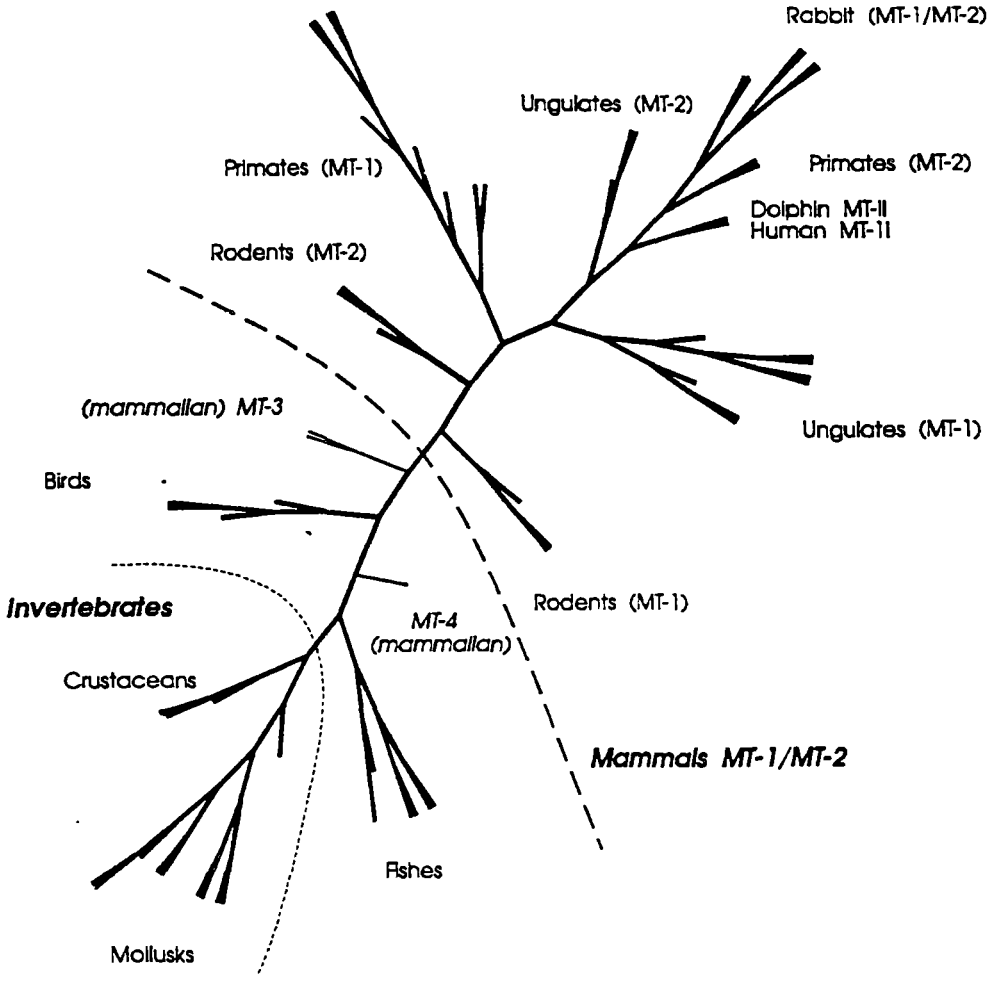


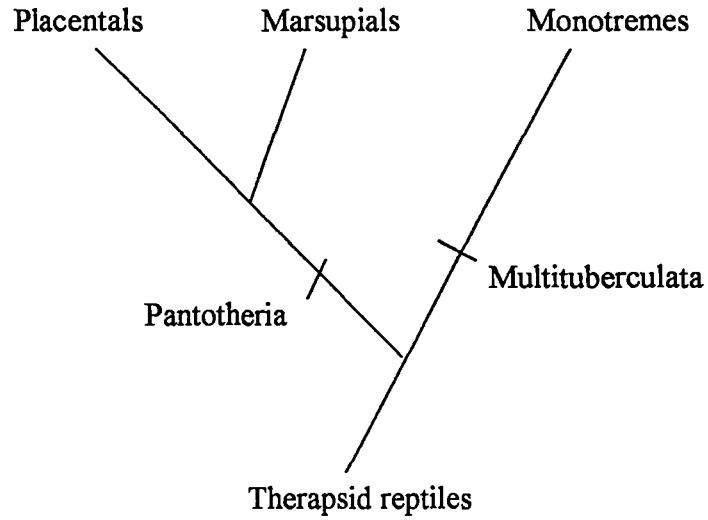
Figure 1.4 Phylogenetic tree of the class I metallothioneins

An unrooted evolutionary tree of the class I metallothioneins calculated by parsimony analysis applied to the amino acid sequences of 62 proteins (modified from Kagi, 1993).

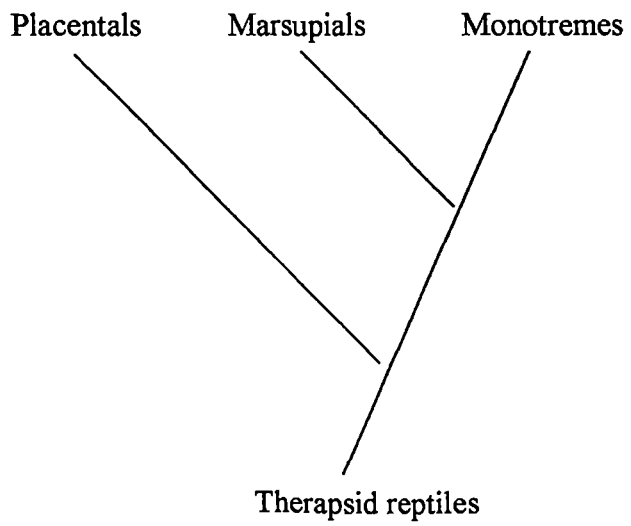
Figure 1.5 Hypotheses of mammalian evolution

Schematic representation of the two major hypotheses of mammalian evolution: (a) the Therian hypothesis and (b) the Marsupionta hypothesis.

a.



b.



al., 1985) and 12S rRNA (Retief *et al.*, 1993) are inconclusive, due to insufficiency of the data. Relatively short protamine P1 amino acid sequences (Gemmell & Westerman, 1994) support the Theria hypothesis, whereas analysis of concatenated mitochondrial protein-coding genes supports the Marsupionta hypothesis (Jancke *et al.*, 1997). DNA hybridisation studies suggest a divergence time between eutherians and marsupials of 107 milllion years ago. A similar estimate based on DNA hybridisation was calculated for the monotreme-therian split, though the slower rate of sequence change in the monotremes is presumed to bias the estimate (Shaw *et al.*, 1993; Kitsch *et al.*, 1997). These latter data do not clarify the veracity of the conflicting hypotheses, and in fact present a third alternative: a simultaneous radiation of the mammals into three subclasses. Mitochondrial sequence data give a divergence date of 130-143 million years ago for the Marsupionta and the Eutheria, with a subsequent separation of the monotremes and marsupials at 116-126 million years ago. The radiation of the eutherians is calculated to have begun 115-125 milion years ago, with the segregation of the myomorph rodents (Jancke *et al.*, 1997). Alternately, calculation of a date for the prototherian-therian split from protamine sequence data place it at 160 million years ago, and a metatherian-eutherian split at 104 million years ago (Retief *et al.*, 1993).

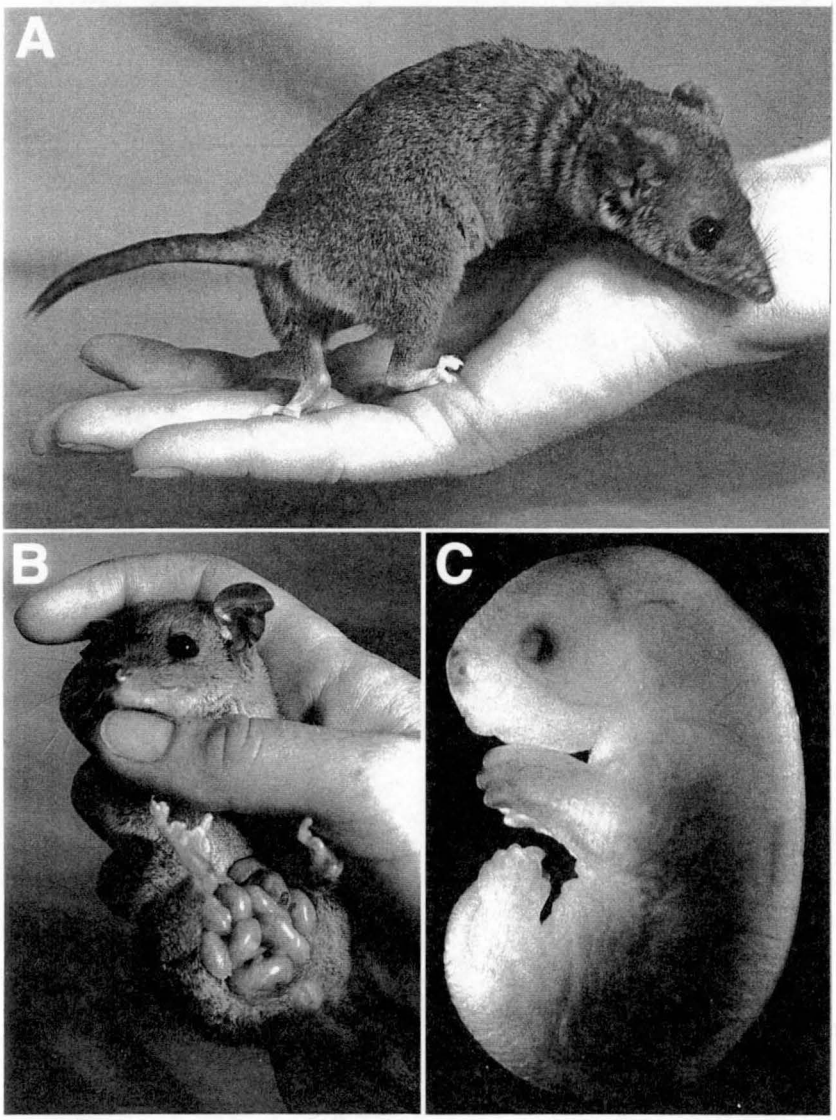
1.3 The Opossum

The opossum *Monodelphis domestica* (class Mammalia; subclass Metatheria or Marsupialia; order Polyprotodonta; suborder Didelphimorphia; family Didelphidae) is a small, pouchless marsupial, native to South America (Fig. 1.6). Marsupials are distinguished from eutherian mammals principally by distinctive reproductive strategies: the young are born extremely small and altricial, and lactation replaces placentation as maternal support during very early development. Apart from these gross differences in reproductive physiology, the marsupial represents a slightly different but equally successful mammalian body plan. Other characters which distinguish the Metatheria from the Eutheria are comparatively subtle, including such traits as typically lower body temperatures and lower standing metabolic rates.

Figure 1.6 The South American Opossum - *Monodelphis domestica*

A. Adult male, 100g.
B. Female opossum with seven-day-old young attached to the abdomenal teats.
C. Two day old pup, crown-rump length = 1cm

(*Photography: Dr. G. Knott*)



The marsupials are important in the study of metallothioneins for two main reasons:

1. marsupials represent a departure from the placental mammals early in the evolution of the class Mammalia, and comparison of the traits common to both subclasses provides insight into the features of the ancestral mammalian form. Comparative studies allow the identification of structure/function relations in mammalian physiology, especially when only slight physiological differences exist.
2. the metallothionein gene family increased in complexity in the mammalian lineage after it diverged from the birds, 310 million years ago. Within this period, the mammalian subclasses also segregated. The events at the marsupial metallothionein locus may clarify the circumstances surrounding the multiple duplications at the eutherian locus. Furthermore, the timing of these duplications could be calculated from a comparison with the marsupial *MT* locus more accurately than is now possible, and with a basis in extraneous and independent palaeontological data.

1.4 The Echidna

The short-beaked echidna *Tachyglossus aculeatus* (class Mammalia; subclass Prototheria; order Monotremata; family Tachyglossidae) (Fig. 1.7), belongs to one of the three extant monotreme species, all native to Australia or Papua-Nuigini. The monotremes are unique among the mammals for their oviparity, which is coupled to other more typically mammalian reproductive traits, including lactation. The monotremes display several "primitive" characters including a reptile-like skeleton. The assumption of primitivity in the echidna, however, is belied by its large and highly convoluted brain (Jerison, 1978). Because they occupy a critical phylogenetic niche at the nexus of mammalian evolution, monotreme data make a valuable contibution to any evaluation of evolution within the mammals.

It has been suggested tht the rate of sequences change in the genes and proteins of *Tachyglossus* is lower than for other mammalian species (Shaw *et al.*, 1993; Kirsch *et al.*, 1997). However, little work has been done on the genetics of the echidna, so this assumption awaits further corroboration.

Figure 1.7 The Australian echidna - *Tachyglossus aculeatus*
(Photography: Dr. G. Knott)

1.5 Aims of the Thesis

The aims of this thesis are:

1. To identify and characterise the metallothionein gene or genes from the marsupuial opossum, *Monodelphis domestica.*
2. To examine the expression of these gene/s through the various tissues of the opossum. Eutherian MT isoforms are defined in part by their tissue-specificity. Any similarities between the tissue-specific expression of marsupial and eutherian MTs is, therefore, investigated.
3. To examine the expression of MT in the liver and brain of the developing marsupial neonate. Because marsupials and eutherians differ most markedly in the physiology of the perinatal young, and this is a time of distinctive MT expression in the eutherian mammals, a comparison of expression profiles may contribute to an understanding of the role of metallothionein during this period of the mammalian life cycle.
4. To construction a phylogenetic history of the mammalian metallothionein gene family, including the members of the marsupials and monotremes. By so doing, it is hoped that a better understanding of the multiple duplication of the mammalian *MT* locus may be possible.

CHAPTER 2: MATERIALS and METHODS

2.1 Buffers and Media

2.1.1 Hybridisation buffers

2.1.1.1 DNA hybridisation buffer (50% formamide)

6g (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]) (HEPES) dissolved in 250ml cold distilled water
5ml 10% SDS
1g ficoll
1g bovine serum albumin (BSA)
1g polyvinylpyrrolidone (PVP)
1ml 0.5M EDTA
75ml 20 x SSC
salmon sperm DNA (0.5mg/ml), boiled for 3 min.
in distilled water

2.1.1.2 RNA hybridisation buffer for oligonucleotide probes

20% formamide
5 x SSC
50mM phosphate buffer, pH6.8
1mM Na-pyrophosphate
0.1% BSA
0.1% ficoll
0.1% PVP
2% SDS
salmon sperm DNA (100 μg/ml), boiled for 3 min.
in distilled water.

The concentration of formamide was varied between 20% and 40%, in accord with the stringency required.

2.1.1.3 RNA hybridisation buffer for cDNA probes

50% formamide
5 x SSC
0.1% ficoll
0.1% BSA
0.1% PVP
1% SDS
10% dextran sulphate
50mM phosphate buffer
salmon sperm DNA (0.5mg/ml), boiled for 3 min.
in distilled water

2.1.1.4 *in situ* hybridisation buffer

(from Penschow & Coghlan, 1994)

600 mM NaCl
50 mM sodium phosphate, pH7.0
5.0 mM EDTA
0.02% ficoll
0.02% BSA
0.02% PVP
0.1% DNA (degraded)
40% formamide (deionized and filtered)
in distilled water

2.1.2 Other buffers

2.1.2.1 10 x MOPS buffer

200mM 3-[N-morpholino]propane-sulphonic acid (MOPS)
50mM sodium acetate
10mM EDTA
Adjusted to pH6.5-7.0

2.1.2.2 0.2M phosphate buffer

3.59g $NaH_2PO_4.2H_2O$

10.93g Na_2HPO_4, anhydrous

dissolved in 500ml distilled water, pH adjusted to 7.2

2.1.2.3 20 x SSC

3M NaCl

0.3M Na-citrate

in distilled water. Adjust to pH7.0

2.1.2.4 STE

0.1M NaCl

10mM Tris-HCl, pH8.0

1mM EDTA

in distilled water. Autoclave.

2.1.2.5 1 x TAE

40mM Tris-acetate

1mM EDTA, pH8.0

2.1.2.6 10 x TBE

121g Tris-base

61.7g boric acid

7.44g EDTA, pH8.3

to 1 litre with distilled water

2.1.2.7 TE

10mM Tris-HCl, pH7.4

1mM EDTA

2.1.3 Culture Media

2.1.3.1 L broth

10g tryptone

5g yeast

5g NaCl

to 1 litre with distilled water. Autoclave.

If necessary, add antibiotics after coolling to a final concentration of:

ampicillin (Boehringer-Mannheim) 50 μg/ml

kanamycin (Boehringer-Mannheim) 50 μg/ml

tetracycline (Sigma) 12.5 μg/ml

2.1.3.2 L agar

To 1 litre of L-broth, add 15g agar. Autoclave.

2.1.3.3 NZY agar

5g NaCl

2g MgSO4.7H_2O

5g yeast

10g casein hydrolysate

15g agar

distilled water to 1 litre, adjust to pH7.5

2.1.3.4 NZY top agarose

5g NaCl

2g MgSO4.7H_2O

5g yeast

10g casein hydrolysate

0.7% agarose

distilled water to 1 litre, adjust to pH7.5

2.1.3.5 lambda diluent

10mM Tris-HCl, pH7.5

10mM $MgCl_2$

0.1mM EDTA

2.1.3.6 SM buffer

5.8g NaCl

2g $MgSO_4.7H_2O$

50mM Tris-HCl (pH7.5

5ml 2% (w/v) gelatin

2.1.4 Other solutions

2.1.4.1 guanidinium thiocyanate

250g guanidinium thiocyanate in 293ml distilled water

17.6ml 0.75M sodium citrate, pH7.0

26.4ml sarcosyl

heated to 65°C and filter sterilised

2.1.4.2 6x DNA loading dye

25% sucrose

6.25mM EDTA

1.25% SDS

0.25% bromophenol blue

2.1.4.3 6x RNA loading dye

50% glycerol

1mM EDTA, pH8.0

0.25% bromophenol blue

0.25% xylene cyanol FF

2.1.4.4 4% paraformaldehyde

4% (w/v) paraformaldehyde dissolved in 500ml distilled water at 60°C, cleared by the addition of several drops of 10M NaOH.
500ml of 0.2M phosphate buffer

2.1.4.5 alkaline agarose

Agarose (0.8g) was dissolved in 72ml distilled water, coolled to 55°C and 8ml 10x alkaline buffer added. Electrophoresis was performed through a thin layer of gel at 100mA.

2.1.4.6 10x alkaline buffer

3ml 5M NaOH
2ml 0.5M EDTA
45ml distilled water

2.1.4.7 alkaline agarose 2x loading buffer

200 µl glycerol
750 µl distilled water
46 µl saturated bromophenol blue
5 µl 5M NaOH

2.1.5 cell lines

1. *E. coli* strain MC1061 (BioRad)
   *Ara*D139 Δ(*ara, leu*)1697 Δ(*lac*)$_{X74}$ *gal*U g*al*K *hsd*R2 *str*A *mcr*A *mcr*B1 Δ((*sr*1-*rec*A)306::Tn*10*)

2. *E. coli* strain XL1-Blue MRF' (Stratagene)
   Δ(*mcrA*)183, Δ(*mcrCB-hsdSMR*-mrr)173, *endA*1 *supE44* thi-1 *recA*1 *gyrA*96 *relA*1 l*ac*[F' *proAB lacI*^{q}ZΔM15 Tn*10* (Tet')]

3. *E. coli* strain XLOLR (Stratagene)

   Δ(*mcrA*)183 Δ(*mcrCB-hsdSMR-mrr*)173 *endA*1 *thi*-1 *recA*1, *gyrA*96, *relA*1, *lac*[F' p*roAB lacI*^{q}ZΔM15 Tn*10* (Tet')]

4. *E. coli* strain Y1090 (from Gibco BRL)

   Δ*lac*U *pro*A+ Δ*lon araD*139 *strA supF* (*trpC*22::Tn*10*)(pMC9)

2.2 Animal Tissues

Tissues from adult and developing opossums were obtained from the animals of a breeding colony maintained at the University of Tasmania by Prof. Norman Saunders. The details of colony management have been described by Fadem et al. (1982) and Saunders et al. (1989). Tissues from LPS-treated opossums were donated by Assoc. Prof. Stewart Nicol, from members of the same colony.

2.3 Cloning

2.3.1 Preparation of vector

Vector DNA was digested with the appropriate restriction enzyme or enzymes. To prevent re-circularization, the 5'-phosphate group was removed from the linearised plasmid with calf-intestinal alkaline phosphatase (Pharmacia). DNA was incubated with 1 unit of alkaline phosphatase for 30 min. at 37°C. The reaction was extracted with phenol/chloroform, and the plasmid precipitated with ethanol.

2.3.2 Preparation of insert

PCR products or restriction fragments were separated electrophoretically on 1% agarose/1xTAE containing ethidium bromide (10 μg/ml). The target band was

excised with a scalpel and purified using Bresaclean (Bresatec, Australia), as described in 2.7.3.

2.3.3 Blunting DNA ends

When necessary, PCR or restriction fragments were blunt-ended using mung bean nuclease (Promega). DNA was incubated with 4.5 units of mung bean nuclease in 20 µl mung bean nuclease buffer (Promega), for 3 hours at room temperature. The DNA fragment was then precipitated with ethanol.

2.3.4 Ligation

Vector (50ng) was combined with a 3x molar excess of insert and 400 units T_4 ligase, in a final volume of 20 µl in ligase buffer (New England Biolabs). The reaction was incubated at room temperature overnight.

2.3.5 Preparation and transformation of competent cells

100ml L-broth was innoculated with an overnight culture of *Escherichia coli* MC1061 cells, and shaken at 37°C until cell density reached $OD_{650nm} = 0.4\text{-}0.5$. Cells were pelleted at 7,000g for 5 min., in pre-coolled tubes and placed on ice. The pellet was resuspended in 20ml ice-cold 50mM $CaCl_2$ (pH7.0) and left on ice for 30 min. before centrifugation at 7,000g for 5 min. Cells were resuspended in 3.33ml of ice-cold 50mM $CaCl_2$ (pH7.0).

Competent cells (200 µl) were combined with 10 µl of the ligation reaction (2.2.4) or 2ng plasmid DNA, and incubated on ice for 40 min. Heat shock was applied at 42°C for 90 seconds. Transformed cells (in 20 µl and 180 µl aliquots) were plated onto L-agar plates containing the appropriate antibiotics, and incubated overnight at 37°C.

2.3.6 Identification of recombinant clones

Bacterial colonies were transferred to nitrocellulose by the method of Grunstein and Hogness (1975). Asymmetrically marked nitrocellulose filters

(Schleicher and Schuell) were lowered onto the surfaces of bacterial plates for 2 min., and the markings copied onto the bottom of the plate. The filter was removed and placed colony-side uppermost on blotting paper soaked in denaturing solution (0.5M NaOH, 0.5M NaCl) for 5 min. The filter was then transferred to blotting paper soaked in neutralising solution (2M NaCl, 0.5M Tris-HCl, pH8.0) for 10 min., before rinsing in 2 x SSC. Residual colonies were wiped from the filters and the filters rinsed again. To cross-link the nucleic acids, the filters were baked under vacuum at 80°C for 2 hours.

Filters were pre-hybridised in 50% formamide DNA hybridisation buffer for one hour at the hybridisation temperature. Heat-denatured probes were added and hybridised overnight, at the appropriate temperature (typically 50-65°C for cDNA, 37-42°C for oligonucleotides probes). Filters were washed in a graded series of buffers: 2 x SSC, 0.1% SDS to 0.2 x SSC, 1% SDS at room temperature. The stringency of washes was increased by raising the temperature as necessary, to remove background radioactivity. Filters were covered in plastic wrap and exposed to autoradiography film (Hyperfilm MP, Amersham) overnight. Positive colonies were sub-cultured in 2ml LB-broth containing 50μg/ml ampicillin, or appropriate antibiotic.

2.3.7 small-scale plasmid preparation

Small-scale extraction of plasmid was performed using alkaline lysis. Once cells are lysed, exposure to alkali denatures bacterial chromosomal DNA, but does not affect supercoiled plasmid DNA. The former may be differentially precipitated, entangled in cell debris, while the latter remains in solution.

2ml bacterial cultures were incubated with shaking at 37°C. Cells were pelleted at high speed for 30s, resuspended in 100 μl ice-cold buffer 1 (50mM glucose, 25 mM Tris-HCl pH8.0, 10mM EDTA) and incubated at room temperature for 5 min. 200 μl fresh buffer 2 (0.2M NaOH, 1% SDS) was added, the solution mixed gently and incubated on ice for 5 min. 150 μl of 5M potassium acetate (pH4.8) was added, the solution mixed by inversion and incubated on ice for a

further 5 min. After high speed centrifugation for 5 min., the supernatant was removed to a clean tube and centrifuged again for 2 min.

Phenol (200 µl) and chloroform (200 µl) were added to the supernatant, vortexed and centrifuged at high speed for 2-5 min. The upper aqueous layer was removed and the nucleic acids precipitated with 2 volumes of absolute ethanol and 0.1 volumes of 3M sodium acetate, with centrifugation at room temperature for 30 min. The pellet was washed once with 70% ethanol, dried under vacuum and resuspended in 20 µl sterile water. RNAse A was added to a final concentration of 0.5 µg/µl and incubated at 37°C for 20 min.

10 µl plasmid DNA was then restricted with enzymes appropriate to the excision of the inserted fragment, in a total volume of 20 µl for 1-2 hours at 37°C. 10 µl sample from each reaction was run on a 1% agarose gel in TBE, at 80V (5V/cm). Plasmid preparations containing insert were identified and the remains of the corresponding bacterial cultures stored at 4°C for future attention.

2.4 Extraction of Total RNA

RNA was extracted by a method modified from Chomczynski and Sacchi (1987). Tissue from newly sacrificed animals was freeze-clamped in liquid nitrogen. Frozen tissue (0.5g) was ground to a fine powder and homogenised in 10ml of guanidinium thiocyanate buffer. To this was added, sequentially: 1ml 2M sodium acetate (pH4.5), 10ml TE-saturated phenol (pH7.0), 2ml chloroform and 40 µl isoamyl alcohol, mixing after each addition. After vortexing for 4 min., the suspension was centrifuged at 10,000g for 20 min. at 4°C. The upper aqueous layer was removed to a clean tube and the RNA precipitated with 10ml isopropanol at -20°C for up to 16 hours. The precipitate was pelleted by centrifugation at 10,000g for 20 min. at 4°C. The pellet was washed in 70% ethanol, air-dried and dissolved in 200 µl sterile, double-distilled water or DEPC-treated water. RNA was snap frozen in liquid nitrogen and stored at -80°C.

All solutions were prepared using double distilled or DEPC-treated water in ribonuclease free glassware (heat-treated to 160°C for 8 hours). All reagents, consumables and pipettes were reserved for RNA work to avoid contamination.

2.5 Northern Hybridisation Analysis

2.5.1 Separation of RNA fragments by denaturing gel electrophoresis

Total RNA was quantified spectrophotometrically at 260nm (1 O.D. = 40 μg/ml). 50 μg RNA, in a volume of 10 μl, was added to 4.5 μl MOPS buffer, 8 μl formaldehyde and 22 μl de-ionised formamide. The solution was vortexed and heated to 65°C for 15 min. to denature the RNA, before coolling on ice. RNA loading dye (4 μl) and 2 μl ethidium bromide (1mg/ml) were added and the mixture vortexed, centrifuged and loaded onto a denaturing gel: 1.2% (w/v) agarose, 2.2M formaldehyde in 1 x MOPS. Electrophoresis of samples was executed in 1 x MOPS buffer, at 30V for 16 hours.

2.5.2 Northern blotting

RNA was adsorbed onto nylon membrane (Zeta-Probe GT, BioRad) by alkaline transfer (0.05M NaOH) for 6-8 hours (Vrati et al., 1987). The membrane was washed twice in 2 x SSC, and baked under vacuum at 80°C for 30 minutes.

2.5.3 Generation of radiolabelled probes

2.5.3.1 α-^{32}P-dCTP labelled cDNA

cDNA fragments were released from vectors with endonuclease restriction and purified as described in 2.2.9. cDNA probes (50ng / 34 μl) were denatured by boiling for 3 min. Probes were labelled with α-^{32}P-dCTP using the T_7 QuickPrime Kit (Pharmacia) in the reaction:

34 μl denatured DNA

10 μl reagent mix

5 μl α-^{32}P-dCTP (3000 Ci/mmol)

1 μl T_7 polymerase

and incubated at 37°C for 30 min. Unincorporated label was removed by size exclusion chromatography on a Sephadex G50 column (2.13.3), or with ProbeQuant Micro Columns (Pharmacia Biotech), as described in 2.13.4. Labelled cDNA was boiled for 5 min. to denature before addition to hybridisation buffer.

2.5.3.2 α-^{32}P-dCTP labelled oligonucleotide probes

Synthetic oligonucleotides were manufactured and desalted by Bresatec Pty. Ltd. (Australia) and AusPep Pty. Ltd. (Australia). Oligonucleotide sequences are given in 2.6.2. 50ng oligonucleotide was labelled by 3' tailing, using terminal transferase and α-^{32}P-dCTP, in the reaction:

1 µl oligonucleotide

2 µl 10 x one-for-all buffer (Pharmacia)

2 µl terminal transferase (Pharmacia)

5 µl α-^{32}P-dCTP (3000 Ci/mmol)

10 µl sterile water

The reaction was incubated at 37°C for 75 min, after which it was placed on ice and the reaction stopped by the addition of 2 µl of 0.5M EDTA (pH8.0). Unincorporated label was removed using ProbeQuant Micro Columns (Pharmacia Biotech), as described in 2.13.4.

2.5.4 hybridisation

Hybridisation was performed in 50% formamide buffer (with dextran sulphate) when cDNA probes were used. When oligonucleotide probes were used, lower concentrations of formamide were used, (20-40%, dependent upon the length of the oligonucleotide) and dextran sulphate omitted. Nylon membranes were sandwiched between hardened filter paper (Whatman #54) and sealed into plastic bags with hybridisation buffer containing denatured, radiolabelled probe. The membrane was hybridised for 16 hours at 37-42°C, determined by the specificity of the probe. Membranes were washed through graduated buffers, typically to 0.2 x SSC, 1% SDS at 37°C. However, washing stringency was increased or decreased by

adjusting the wash temperature, with temperatures up to 65° used to remove background radioactivity. Membranes were rinsed in 0.2 x SSC, wrapped in plastic wrap and exposed to autoradiographic film (2.4.5).

2.5.5 autoradiography

Membranes were exposed to autoradiography film (Hyperfilm MP, Amersham) which had been pre-flashed as decribed in 2.13.6. Film and filters were exposed in cassettes, between two intensifying screens, at -80°C.

2.5.6 removing probes from filters

To remove radioactive probes from northern (and Southern) blots, membranes were shaken in stripping buffer (0.5% SDS; 0.1% x SSC) at >95°C for 15-20 min. The process was repeated once, and membranes then rinsed for 5-10 min. in 0.2 x SSC.

2.6 Southern Hybridisation Analysis

2.6.1 Southern blotting

DNA was separated electrophoretically on non-denaturing agarose and was transferred onto nylon membrane (Zeta-Probe GT, BioRad) by alkaline blotting (0.4m NaOH) for 10-16 hours. The membrane was rinsed in 2 x SSC and baked under vacuum at 80°C, for 30 min.

2.6.2 hybridisation

The membrane was enclosed in a plastic bag with 50% formamide DNA hybridisation buffer and pre-hybridised at the appropriate temperature for 1 hour. Denatured radiolabelled probe was added and the membrane hybridised at a temperature defined by the type of probe (cDNA or oligonucleotide) and the requisite stringency. Membranes were washed to a stringency of 0.2 x SSC, 1% SDS at room temperature, and the temperature increased in accord with the stringency required. Temperature and washing regimes are described for each

experiment in the text. Membranes were rinsed in 0.2 x SSC, wrapped in plastic wrap and exposed to autoradiographic film as described in 2.4.5.

2.7 Probes

2.7.1 cDNA probes

mMT3 - a 274bp cDNA fragment, amplified from mouse brain RNA by RT-PCR, using mouse *MT3* sense (5'-TGGATATGGACCCTGAGACC-3') and antisense (5'-CCTTGGCCCCCTCTTC-3') PCR primers (Holloway, 1996).

sMT3 - a 147bp *Hae*III fragment (bases 12-158 of the coding sequence) of the sheep MT3 cDNA (Holloway, 1996).

MdMT - a 90bp cDNA fragment (bases 46 to 135 of the coding sequence), amplified from opossum liver RNA by RT-PCR, using mouse *MT1* sense (5'-GACCCCAACTGCTCCTGCT-3') and antisense (5'-GCAGCCCTGGGCACACTTG-3') PCR primers, as described in 3.2.1.1.

mMT4 - a 286bp cDNA fragment (containing the complete coding sequence), amplified from mouse tongue epithelium RNA by RT-PCR, using the mouse *MT4* sense (5'-CCgagctcCAGCCTCCCTTTCTTAGCTG-3', lower case letters represent incorporated restriction site) and antisense (5'-CCtctagaCCCTTGACTCAGGTACTGTG-3') PCR primers, as described in 3.2.3.2.

2.7.2 oligonucleotide probes and primers

Oligonucleotide probe sequences were analysed with the computer program Oligo 5.0 (Rychlik, 1994) to preclude hairpin loops or interoligonucleotide duplices, and to establish hybridisation parameters.Oligonucleotides were synthesised by Bresatec Pty. Ltd. (Australia) or Auspep Pty. Ltd. (Australia).

cDNA-specific oligonucleotide probes were designed to the 3' untranslated regions of each of five opossum metallothionein cDNA molecules.

* MdMTB1: 5'-TGAGTTGTGTTTTCTAA-3'
* MdMTB3: 5'-TCAATCCACAGGCATTTA-3'
* MdMTL2: 5'-CATTTTTCTTTGCCTGTA-3'
* MdMTL3: 5'-CACCTCCTCATTCCTGTA-3'
* MdMTx5: 5'-TTCAGATGTGGCTTTTTATACACTGTAGAT-3'

A degenerate oligonucleotide probe was designed to the consensus sequence of documented mammalian *MT3* sequences, as described in Fig. 3.11.

* MT3 probe: 5'-AGTAGGRCAGGGGCAGGTCTC-3' where R = A or G

An oligonucleotide probe designed to sites 1750-1776 of the 18S ribosomal RNA molecule, conserved in all vertebrates (Hillis & Dixon, 1991), was used as a control when probing northern blots.

* 18S rRNA probe: 5'-CAATCGGTAGTAGCGACGGGCGGTGTG-3'

Sequencing primers used with pUC19 vector:

* M13 universal primer: 5'-GTAAAACGACGGCCAGT-3'
* M13 reverse primer: 5'-CAGGAAACAGCTATGAC-3'

2.8 Reverse Transcriptase - Polymerase Chain Reaction

2.8.1 reverse transcription

Total RNA (1 μg) was reverse transcribed under the following conditions:

4 μl 5x First Strand Buffer (250mM Tris-HCl, pH8.3; 375mM KCl; 15mM $MgCl_2$)

2 μl dithiothreitol (1mM stock, 100μM final concentration)

4 μl dNTPs (2.5mM stock, 0.5mM final concentration of each)

1 μl random hexamer, $pd(N)_6$ (10 μg/ml final concentration)(Pharmacia)

1 μg RNA

1 μl RNAsin (40U/μl)(Promega)

200 units M-MLV reverse transcriptase (Gibco BRL)

DEPC-treated water to a final volume of 20 µl.

The reaction was incubated for one hour at 37°C. Reverse transcriptase was inactivated by heating to 99°C for 5 min and the reaction products stored at -20°C.

2.8.2 polymerase chain reaction

cDNA was amplifed by PCR in the following reaction:

2.5 µl 10x PCR buffer (100mM Tris-HCl, 15mM $MgCl_2$, 500mM KCl, pH8.3)

2.5 µl dNTPs (2.5mM stock, 250µM final concentration)

1 µl forward primer (10µM stock, 0.04µM final concentration)

1 µl reverse primer (10µM stock, 0.04µM final concentration)

0.5 µl *Taq* DNA polymerase (2.5 units, Boehringer Mannheim)

10ng cDNA

sterile distilled water to a final volume of 25 µl

A Perkin Elmer Cetus DNA thermocycler was used for incubation of the reaction, and the cycling parameters:

	95°C, 2 min	
denaturation	95°C, 1 min	⎫
annealing	60°C, 1 min	⎬ 35 cycles
extension	72°C, 1 min	⎭
	72°C, 7 min	

Variations in annealing temperature were employed where necessary, and these are described within the text.

2.8.3 purification of PCR product

The following procedure was used to purify PCR product, using the Bresa-Clean™ Nucleic Acid Purification Kit (Bresatec, Australia).

Products of the polymerase chain reaction were separated electrophoretically on 1% agarose in TAE, with ethidium bromide (final concentration of 0.5 µg/ml). The amplified fragment was excised with a scalpel under UV transillumination, and

dissolved in BresaSalt™ at 55°C. Silica milk was added, and the solution inverted continuously for 5 min. at room temperature. Silica-bound DNA was pelleted, resuspended in ice-cold BresaWash™ and pelleted again. BresaWash™ was removed, and the DNA eluted from the silica in MQ.H_2O at 55°C.

2.8.4 PCR primers

Primer	Primer Sequence
MT1 forward	5'-GACCCCAACTGCTCCTGCT-3'
MT1 reverse	5'-GCAGCCCTGGGCACACTTG-3'
MT2 forward	5'- ATGGACCCCAACTGCTCCTGTGCCTCC-3'
MT2 reverse	5'-GGTCTATTTACACAGATGTGGGGACCC-3'
Degenerate *MT3* forward	5'-ATGGAYCCNGARAC-3'
Degenerate *MT3* reverse (Palmiter *et al.*, 1992)	5'-CAYTTYTCNGCYTC-3'
*MT3Pst*I forward	5'-CCctgcagTGGATATGGACCCTGAGACC-3'
*MT3Sac*I reverse	5'-CCgagctcGCTGCAYTTYTCNGCYTC -3'
m*MT4Sac*I forward	5'-CCgagctcCAGCCTCCCTTTCTTAGCTG-3'
m*MT4Xba*I reverse	5'-CCtctagaCCCTTGACTCAGGTACTGTG-3'
MT4A forward	5'-ATHTGYATNTGYGGNGAYAAT-3'
MT4B forward	5'-ATHTGYATNTGYGGNGAYAAC-3'
MT4reverse	5'-YTTNCKRCANGTYTTRCA-3'

Y = C, T H = A, C, T K = G, T

R = A, G N = any nucleotide

Letters in lower case represent restriction recognition sites incorporated into the primers.

2.9 DNA Sequencing

2.9.1 Sanger dideoxy sequencing

The Sanger dideoxy chain termination sequencing method was used as outlined in Sambrook *et al.*, 1989. Double stranded fragments were cloned into pUC19 vector, and the constructs purified using Qiagen Plasmid Mini Kit™ (QIAGEN), according to manufacture's instructions. Cloned fragments were sequenced using Sequenase version 2 (USB) in accord with recommended protocols, using the M13 universal and reverse primers (2.6.2). Fragments incorporating α-^{35}S-dATP were separated electrophoretically through 6% polyacrylamide gels and detected autoradiographically.

2.9.2 automated sequencing

The ABI PRISM™ Dye Primer Cycle Sequencing Reaction Kit (Perkin Elmer Cetus) and the ABI PRISM™ Dye Terminator Cycle Sequencing Reaction Kit (Perkin Elmer Cetus) were used for DNA sequencing. Double stranded fragments in pUC19 or pBK-CMV were purified using the Qiagen Plasmid Mini Kit™ (QIAGEN) and the cycle sequencing reaction kits used according to manufacturer's instructions. M13 universal and reverse primers were used in conjunction with the ABI PRISM™ Dye Terminator Cycle Sequencing Reaction Kit. An ABI Prism 377 DNA Sequencer (Perkin Elmer Cetus) was used for automated sequencing.

2.10 Construction of ZAP Express cDNA Libraries

2.10.1 isolation of polyA$^+$ mRNA

Total RNA was isolated from the liver and whole brain of the opossum, *M. domestica*, as described in 2.3. PolyA$^+$ mRNA was purified from total RNA using

the Dynabeads mRNA Purification Kit (Dynal). PolyA$^+$ mRNA was then used in the construction of a cDNA library.

100 µl RNA (75µg) was heated to 65°C for 2 min and added to 1.0mg of oligo(dT)$_{25}$ Dynabeads suspended in 2x binding buffer (20mM Tris-HCl, pH7.5; 1M LiCl, 2mM EDTA) for 5 min. The suspension was placed in the magnetic particle concentrator (Dynal MPC™-E1) for 30 sec., and the supernatant aspirated. Beads were washed twice with 200 µl washing buffer (10mM Tris-HCl, pH7.5; 0.15M LiCl; 1mM EDTA). PolyA$^+$ mRNA was eluted in 20 µl of 2mM EDTA (pH7.5) at 65°C. The magnetic beads were removed immediately. The polyA$^+$ mRNA was checked by electrophoresis of an aliquot through a 1% agarose gel in TBE, and visualised with ethidium bromide.

2.10.2 cDNA synthesis and addition of *Eco*RI adaptors

All the reaction components used in the preparation of the cDNA libraries were supplied in the ZAP Express™ cDNA Synthesis Kit (Stratagene), unless otherwise specified.

The first strand of cDNA was synthesised in the reaction:

5.0 µl 10x first strand synthesis buffer

3.0 µl first strand methyl nucleotide mixture

2.0 µl linker-primer (1.4 µg/µl)

1.0 µl RNAse Block Ribonuclease Inhibitor (40U/µl)

37.5 µl polyA$^+$ mRNA (3 µg)

The linker-primer is a 50-mer oligonucleotide of sequence:

5'-GAGAGAGAGAGAGAGAGAGAACTAGTctcgagTTTTTTTTTTTTTTTTTT-3'

Lower case letters mark the *Xho*I restriction site, used subsequently in cloning the cDNA. Because methylated dCTP is used in first strand synthesis, the DNA is protected from digestion in subsequent cloning steps.

Template (mRNA) and primer were allowed to anneal at room temperature for 10 min. 1.5 µl MMLV-RT (50U/µl) was added and the reaction incubated at 37°C for 1

hour. A radioactive control was simultaneously prepared to monitor the synthesis of the cDNA: 5 µl of reaction mix was added to 0.5 µl α-^{32}P-dATP, and incubated.

Second strand synthesis was performed by the sequential addition of the following components on ice:

45.0 µl (non-radioactive) first strand synthesis reaction

20.0 µl 10x second strand buffer

6.0 µl second strand nucleotide mixture

88.9 µl sterile water

2.0 µl α-^{32}P-dATP

2.0 µl RNAse H (1.5U/µl)

11.1 µl DNA polymerase I (9U/µl)

The nick translation reaction was incubated at 16°C for 2.5 hours, and placed immediately on ice. Excess non-methylated dCTP was supplied to the reaction, to ensure that the linker-primer sequence remained unmethylated, and therefore accessible to later restriction.

To blunt-end the cDNA fragment, 23.0 µl blunting dNTP mix and 2.0 µl of cloned *Pfu* DNA polymerase (2.5U/µl) were added to the second strand synthesis reaction. The reaction was incubated at 72°C for exactly 30 min., and then extracted once with phenol-chloroform, and once with chloroform. The cDNA was precipitated with 20 µl 3M sodium acetate and 400 µl absolute ethanol, at -20°C for 16 hours.

To add *Eco*RI adaptors, the pelleted cDNA was resuspended in 9.0 µl *Eco*RI adaptor mix, and incubated at 4°C for 30 min. To monitor second strand synthesis, 1.0 µl of the reaction was removed and stored at -20°C for later assessment. To the reaction were added:

1.0 µl 10x ligase buffer

1.0 µl 10mM rATP

1.0 µl T_4 ligase (4U/µl)

Reaction was incubated at 4°C for 48 hours. The ligase was then heat-inactivated at 70°C for 30 min.

To phosphorylate the *Eco*RI ends, the following reactants were added sequentially:

1.0 µl 10x ligase buffer

2.0 µl 10mM rATP

6.0 µl sterile H_2O

1.0 µl T_4 polynucleotide kinase

and the reaction incubated at 37°C for 30 min. The kinase was heat-inactivated at 70°C for 30 min.

*Xho*I digestion of the cDNA was performed with the addition of 28.0 µl of *Xho*I buffer supplement and 3.0 µl *Xho*I (40U/µl), followed by incubation at 37°C for 1.5 hours. After coolling, 5.0 µl of 10x STE buffer was added to the reaction in preparation for size fractionation

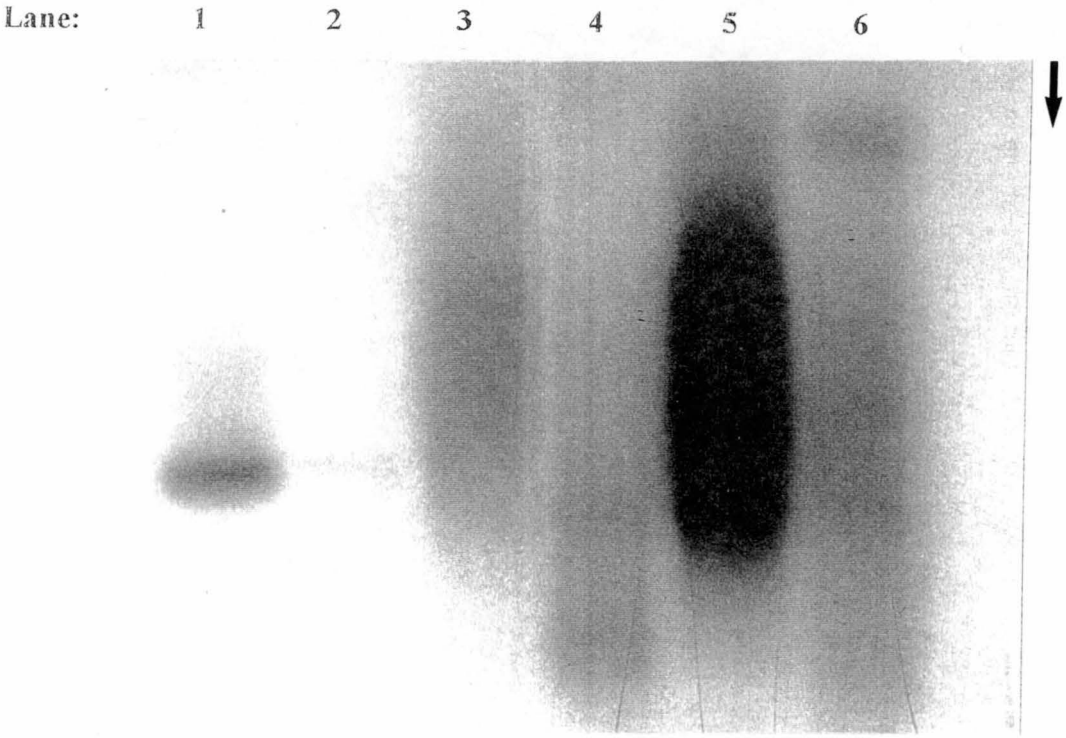
First and second strand synthesis was monitored by gel electrophoresis as described in Fig. 2.1.

2.10.3 size fractionation

All reagents other than those required for gel electrophoresis were supplied in the ZAP Express™ cDNA Synthesis Kit from Stratagene.

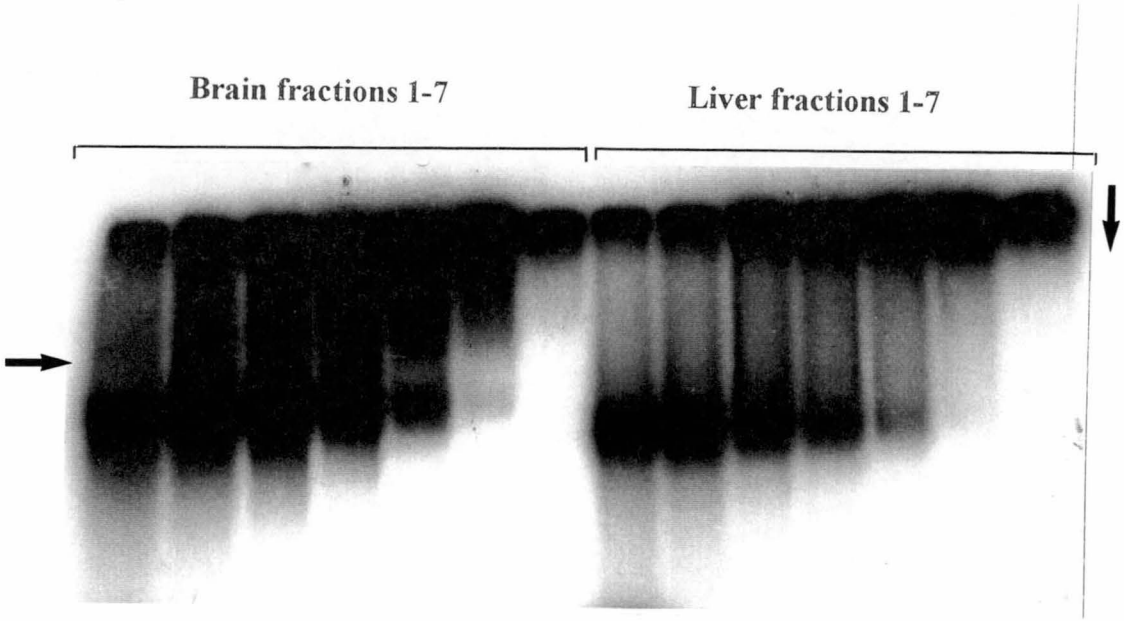
Size fractionation of cDNA was performed using a Sephacryl S-500 spin column. The column was prepared by pipetting sephacryl into a 1 ml syringe, which had previously been plugged with cotton wool. The column was centrifuged at 400g for 2 min., refilled and the centrifugation repeated. The column was washed twice with 300 µl of STE buffer. cDNA was pipetted onto the top of the column, and the column spun at 400g for 2 min. The first fraction was collected. Subsequent fractions were collected by adding 60 µl STE buffer to the column and spinning as before. Seven fractions were collected in this way and 10% of each fraction run on a 5% (29:1) non-denaturing polyacrylamide gel, to establish the size range of the cDNA within each fraction (Fig. 2.2).

Figure 2.1: First and second strand synthesis for cDNA libraries



Poly(A)+ RNA (3μg) was isolated from opossum liver and whole brain to construct cDNA libraries using the Stratgene ZAP Express vector system.
First and second strand synthesis of the cDNA was monitored by electrophoresis of an aliquot of the reactions through 1% alkaline agarose at 100mA for 1.5 hours. The gel was partially dehydrated with pressure and exposed to pre-flashed film for 1.5 hours at -80° with intensifying screens. Test poly(A)+ RNA provided by Stratgene was used as control. Test RNA first strand – 10% of reaction, runs in a tight band at 1.8kb. Second strands are, as usual, 5-10% intensity of first strands.
Lane 1: test RNA, first strand
Lane 2: test RNA, second strand
Lane 3: brain RNA, first strand
Lane 4. brain RNA, second strand
Lane 5: liver RNA, first strand
Lane 6. liver RNA, second strand

Figure 2.2: Size fractionation of cDNA used in the construction of libraries



Products of reverse transcription were size fractionated through S-500 sephadex spin columns. 10% of each fraction was run on a 5% non-denaturing acrylamide gel at 100V for 2 hours. The gel was dried under vacuum for two hours at 60°C and exposed to film overnight at -80°C. Inclusion of DNA markers allowed the size of DNA fractions to be estimated, and the arrow indicates a length of 500bp.
Lanes1-7: Fractions 1-7 of brain cDNA
Lanes 8-14: Fractions 1-7 of liver cDNA

cDNA fractions 1-5 were extracted with phenol-chloroform, then with chloroform alone, and then precipitated with two volumes of ethanol at -20°C overnight. cDNA was pelleted by centrifugation at 13000g for 1 hour at 4°C, washed with 200 µl of 70% ethanol and dried under vacuum. cDNA was resuspended in sterile water and combined into a total volume of 10.5 µl. Size fractionation of cDNA fragments is described in Fig. 2.2.

2.10.4 quantitation of cDNA by ethidium bromide plate assay

To quantitate the cDNA before ligating into the library vector, an ethidium bromide plate assay was used. Plates of 0.8% agarose in 1x TAE buffer were supplemented with ethidium bromide (etbr) to a final concentration of 1µg/ml. A DNA standard (pBR322 - Pharmacia Biotech) of known concentration was serially diluted in 100mM EDTA from 10 - 200 ng/µl. 0.5 µl of each diluted standard and of each sample of cDNA was dotted onto the surface of an etbr plate and allowed to absorb for 10-15 min. at room temperature. The plate was inverted over a UV transilluminator and photographed using Polaroid film. An estimate of the concentration of cDNA samples was made from a fluorescence comparison with pBR322 samples of known concentration.

2.10.5 cloning cDNA into ZAP Express™ vector

All reagents were supplied with the ZAP Express™ cDNA Synthesis Kit (Stratagene). The following reactants were combined:

2.5 µl cDNA (~100ng)

0.5 µl 10x ligase buffer

0.5 µl 10mM rATP (pH7.5)

1.0 µl ZAP Express vector (1µg/µl)

0.5 µl T_4 ligase

and incubated at 12°C overnight. A test ligation was run in parallel using the test insert provided.

2.10.6 packaging the libraries

The libraries were packaged using the ZAP Express™ cDNA Gigapack® II Gold Cloning Kit (Stratagene). 2.5 µl of cDNA-vector ligation reaction was added to 10 µl of freeze-thaw extract (provided). Sonic extract (15 µl) was added with gentle mixing and the reaction incubated at 22°C for 2 hours. SM buffer (500 µl) and 20 µl chloroform were added. After brief centrifugation to sediment debris, the supernatant was decanted and stored at 4°C. As a positive control, 0.2 µg λcI857 *Sam7* wild-type control DNA (supplied with the ZAP Express™ cDNA Gigapack® II Gold Cloning Kit) was packaged.

2.10.7 plating the libraries

XL1-Blue MRF' cells (supplied with the ZAP Express™ cDNA Gigapack® II Gold Cloning Kit from Stratagene) were grown to a density of $OD_{600nm} = 0.5$. These cells (200 µl) were then added to 1.0 µl of packaged recombinant phage, and also to 1.0 µl of a 1:10 dilution of recombinant phage. Phage were allowed to adhere to cells at 37°C for 15 min. The following components were then added:

3ml NZY top agar (48°C)

15 µl 0.5M IPTG

50 µl X-gal (250mg/ml in DMF)

and plated immediately onto NZY agar plates, with incubation overnight at 37°C. Packaged wild-type lambda control DNA was plated similarly, though with the VCS257 cells provided, rather than XL1-Blue MRF'.

2.10.8 amplification of the libraries

Because primary libraries are unstable, one round of amplification was performed on each library. Aliquots of the packaged recombinant phage, containing 50,000 plaque-forming units, were mixed with 600 µl of XL1-Blue MRF' cells at a density of $OD_{600nm} = 0.5$ and incubated at 37°C for 15 min. Melted NZY top agar (6.5 ml) was added and the mixture poured onto fresh NZY agar plates. The plates were incubated for 6-8 hours, until plaques were visible but less than 1 mm in

diameter. SM buffer (10 ml) was applied to the surface of the plates and the plates stored overnight at 4°C with gentle shaking, to allow diffusion of phage into the buffer. Phage were recovered by removing the buffer from plates, adding 5% (v/v) chloroform and incubating at room temperature for 15 min. Cell debris was removed by centrifugation at 500g for 10 min. and the phage stored at 4°C. Titre was calculated from serial dilutions of stock library, plated as described above.

2.11 Screening ZAP Express cDNA Libraries

2.11.1 screening

600 µl XLBlue1-MRF' cells (OD_{600nm} = 0.5) were incubated with 15,000 pfu at at 37°C for 15 min. 6.5ml NZY top agarose (48°C) was added and the mixture plated onto a 150mm NZY agar plate. Plates were incubated at 37°C overnight. After pre-chilling for 2 hours at 4°C, plates were overlain with nitrocellulose membranes for 2 min. (Orientation marks were applied to both agar and plate with waterproof ink). A duplicate filter was applied to the plate for 4 min. Filters were then submerged in denaturing solution (1.5M NaCl; 0.5M NaOH) for 2 min, followed by submersion in neutralisation solution (1.5M NaCl; 0.5M Tris-HCl, pH8.0) for 5 min. Filters were then rinsed in 0.2M Tris-HCl (pH7.5); 2 x SSC for 30 sec. After briefly blotting on Whatman's 3MM, filters were baked under vacuum at 80°C for 2 hours. Agar plates were stored at 4°C.

Filters were pre-hybridised in 50% formamide DNA hybridisation buffer at the appropriate temperature (specified in the text) for one hour. Denatured probes were added and the hybridisation continued overnight. Filters were washed to a stringency of 0.1 x SSC, 1% SDS at room temperature, and the temperature increased in accord with the stringency required. Filters were wrapped in plastic wrap and exposed to autoradiography film overnight at -80°C, with intensifying screens. Positive plaques were located on plates, excised from the agar, suspended in 1 ml SM buffer containing 20 µl chloroform, and vortexed.

These samples were diluted and titred as before, and the process repeated in secondary and tertiary screens, until single positive plaques could be identified and picked with ease. A single plaque in 1 ml SM buffer represents approx. 1 x 10^6 pfu.

2.11.2 single clone excision

Plaques from the tertiary screen were suspended in 500 µl SM buffer containing 20 µl chloroform, vortexed and incubated at room temperature for 1-2 hours. In a Falcon 2059 tube were combined:

200 µl XLBlue1-MRF' cells (OD_{600nm} = 1.0, in 10mM $MgSO_4$)

250 µl phage stock

1 µl ExAssist helper phage (>1 x 10^6 pfu/µl) supplied by Stratagene

The suspension was incubated for 15 min at 37°C.

To the phage mixture was added 3 ml NZY broth and the mixture was incubated for 2.5-3 hours at 37°C with shaking. The tube was heated to 65-70°C for 20 min., then centrifuged at 1000g for 15 min. The supernatant, containing excised pBK-CMV phagemid vector packaged as filamentous phage, was stored at 4°C.

To plate excised phagemids, 200 µl fresh XLOLR cells (resuspended to OD_{600nm} = 1.0 in 10mM $MgSO_4$) were combined with 100 µl and 10 µl of phage supernatant, and incubated at 37°C for 15 min. 300 µl NZY broth was added and the incubation continued for a further 45 min. Cells (200 µl) were plated on LB agar supplemented with kanamycin (50 µg/ml) and incubated overnight at 37°C.

2.12 Screening λgt10 cDNA Library

The echidna liver λgt10 cDNA library, kindly donated by Prof. G. Schreiber (University of Melbourne, Australia), was serially diluted in λ diluent and the titre measured in Y1090 cells. 14,000 pfu were plated with XLBlue1-MRF' cells in NZY agarose onto each of four 150mm NZY agar plates, and screened as for the ZAP Express cDNA Libraries, described in section 2.10. Hybridisation of filters with the MdMT cDNA probe was performed at 55°C in 50% formamide DNA hybridisation buffer, and filters were washed in 0.2 x SSC, 1% SDS at 37°C for 15 min.

Positive plaques retained through to the tertiary screen were purified by a method modified from Sambrook *et al.*, 1989. Positive plaques were picked and stored in 1ml SM buffer with 20 µl $CHCl_3$. A 50ml overnight culture of Y1090 was prepared in LM broth (LB broth supplemented with 0.2% maltose and 10mM $MgSO_4$), and innoculated with aliquots of plaque suspension (4µl, 40 µl, 400 µl). Cultures were incubated with shaking at 37°C till lysis or near-lysis, as detected by the chloroform test (9-12 hours). 0.5ml chloroform was added and cultures shaken for a further 10 min. To the culture was added:

5 µl DNAse (10 mg/ml)

5 µl RNAse A (10 mg/ml)

25 µl 1M $MgCl_2$

with overnight incubation at 37°C.

NaCl (2.29g) was dissolved in the lysed cultures, to a final concentration of 1M, and placed on ice for1 hour. Cultures were centrifuged at 11,000g for 20 min. at 4°C. To the supernatant was added 5g solid polyethylene glycol (PEG) 8000, which was dissolved by inverting the solution continuously for some time at room temperature. The solution remained at 4°C for several days before centrifugation at 11000g for 10 min. at 4°C. The pellet was re-dissolved in 10 ml TE, to which was added:

50 µl pronase (10 mg/ ml - self-digested)

500 µl 10% SDS (final concentration of 0.5%)

and the solution was incubated overnight at 37°C.

The samples were extracted once with phenol/chloroform and centrifuged at 11,000g for 10 min, then once with chloroform and re-centrifuged. The supernatant was precipitated with 0.1 volumes of 3M sodium acetate and 2 volumes of absolute ethanol. DNA was spoolled and redissolved in sterile water.

λ DNA was restricted with *Eco*RI and resulting fragments gel purified before sub-cloning into pUC19.

2.12.1 *the chloroform test*

To 1 ml of incubated culture, 2 drops of chloroform were added, followed by a 10 min. incubation at 37°C. The culture was then compared with a control sample

to which no chloroform had been added. If the former culture appeared less turbid than the latter, it was presumed that infection of the original culture was near completion, and had been driven to lysis by the addition of chloroform. If no difference was apparent, incubation of the original culture was continued.

2.13 *in Situ* Hybridisation

2.13.1 tissue preparation

Tissue samples of approximately 2mm thickness were fixed for 12-18 hours in 4% (w/v) paraformaldehyde freshly dissolved in 0.1M phosphate buffer. Samples were then rinsed three times in 0.1M phosphate buffer (pH7.2), rinsed briefly in distilled water and dehydrated through a graded series of alcohol from 70%, with a minimum of 1 hour in each. Tissues were embedded in paraffin (MP 56°), 0.7μm sections cut and floated onto gelatinised slides. Sections were de-waxed in xylene, rehydrated through graded alcohols and the slides transferred to TE (50mM Tris-HCl, pH7.5; 5mM EDTA). All samples were prepared and processed in triplicate.

2.13.2 protease treatment

An aliquot of 625 μl pronase E (40mg/ml - pre-digested for 4 hours at 37°C) was added to 200 μl pre-warmed TE buffer, to a final concentration of 125 μg/ml. Slides were immersed in pronase/TE solution at 37°C for 10 min. and rinsed twice in 0.1M phosphate buffer.

Slides were immersed in 4% (w/v) paraformaldehyde buffer for 10 min. at room temperature to post-fix samples. Slides were then washed twice in 4 x SSC (0.6M NaCl; 0.06M sodium citrate).

2.13.3 prehybridisation

Slides were placed into hybridisation buffer pre-warmed to 37°C for 2-5 hours. They were then washed vigorously three times in absolute ethanol to remove hybridisation buffer, before air-drying. Slides were stored overnight, if necessary, at

4°C in an airtight opaque box, with a thin layer of alcohol on the bottom to humidify the chamber.

2.13.4 probe preparation

cDNA probes were purified as described in section 2.4.3.1, ethanol precipitated and reconstituted to a concentration of 100 ng/250 µl in hybridisation buffer. Probes were boiled for 3 min. immediately prior to use.

2.13.5 hybridisation

Tissue sections on each slide were covered with denatured probe and a cover slip. Slides were placed in a hybridisation box, humidified by a thin layer of hybridisation buffer in the bottom. Rows of slides were covered with plastic wrap and incubated overnight at 45°C.

Slides were rinsed in 2 x SSC to remove cover slips, then incubated in pre-warmed 1 x SSC at 40°C, with gentle shaking. Slides were rinsed in three changes of absolute ethanol, to remove salt and then air-dried.

2.13.6 x-ray film autoradiography

Dried slides were exposed directly to x-ray film (Hyperfilm MP, Amersham), overnight at room temperature. Cassettes were weighted heavily to maintain contact. Autoradiographs were developed as usual and potentially informative slides identified.

2.13.7 liquid emulsion autoradiography

Liquid emulsion (Ilford K5 Nuclear Research Emulsion) was diluted with two volumes of distilled water and incubated at 37°C for 3 hours. Slides were immersed in diluted emulsion, placed in metal racks and incubated in light-proof metal tins over anhydrous silica gel for one half-life of the isotope, which in the case of ^{32}P is 14 days. After this time, slides were immersed in D19 (Kodak) developer

for 2 min., rinsed in distilled water, immersed in fixer for 5 min. and rinsed again in distilled water.

2.13.8 counterstaining and dehydration

Sections were stained in haematoxylin solution (Sigma), then dehydrated through a graded series of alcohols and two rinses in xylene. Cover slips were affixed with DPX mountant (Koch-Light Laboratories, Ltd.) and left to air dry overnight.

2.13.9 photography

Tissue samples were photographed using an Olympus BX50 microscope, and the Olympus PM20 automatic photomicrographic system. Films used were AgfaPan 35 mm APX25 and APX400 for black and white photographs, with a green filters during bright field exposures. Kodak Ektachrome Professional 64T was used for colour slides, with neutral density filters LBD and ND6 for bright field exposures.

2.14 Miscellaneous Procedures

2.14.1 preparation of TE saturated phenol

Phenol was melted at 65°C, then 40% (v/v) TE buffer added. The phenol was shaken vigorously for 5 min. The phases were allowed to separate overnight at 4°C and the aqueous layer discarded. The procedure was repeated with 25% (v/v) TE buffer and most of the aqueous layer removed. 8-hydroxyquinolone was added to a final concentration of 0.1%.

.

2.14.2 preparation of 6% polyacrylamide sequencing gel

A 40% (w/v) stock of acrylamide and bisacrylamide (20:1) was prepared in distilled water, and stored at 4°C in a light-proof container. A 6% ployacrylamide gel was prepared with the following constituents:

42g urea

20ml 10x TBE

15ml 40% acrylamide stock

distilled water to a final volume of 98.2ml

The gel mixture was filtered through a 0.45 μm filter and degassed for 20 min. Freshly prepared 10% ammonium sulphate (0.8ml) and 80 μl TEMED (N,N,N',N'-tetramethylethylenediamine - Sigma) were added immediately prior to pouring, to initiate polymerisation. The gel was allowed to set overnight, wrapped in plastic wrap. The loaded gel was pre-run at 1500V for 30 min. Loaded gels were run at 1500V for 3.5-6 hours. Air-dried gels were exposed to autoradiography paper (Hyperpaper, Amersham) for 1-3 days.

2.14.3 preparation of sephadex columns

Sephadex G50 was added to sterile distilled water, washed several times and then equilibrated with STE buffer. The slurry was autoclaved for 10 min. and stored at 4°C. The column was prepared by filling a clean 0.5cm diameter glass column with Sephadex to 1cm in height.

2.14.4 purifiying radioactive probes through spin columns

To the reaction products of the oligonucleotide probe labelling reaction (2.4.3.2) was added 28 μl column buffer (10 mM Tris-HCl, pH7.5; 1mM EDTA, 100 mM NaCl). The reaction products of the cDNA probe labelling reaction (2.3.4.1) were divided equally into two tubes, and to each was added 25 μl column buffer. ProbeQuant Micro Columns (Pharmacia Biotech) were prepared by vortexing sephadex to mix, prior to centrifugation for 1 min. at 735g. The radioactive sample was then added to the column and the column centrifuged again at 735g for 2 min. cDNA probe samples were then recombined. For use with northern or Southern blotting, probes were then boiled for 3 min. before adding to hybridisation buffer. For use with *in situ* hybridisation, probes were ethanol precipitated with 3M sodium acetate and and 50 μg tRNA, and the pellet redissolved in 10 μl sterile water.

2.14.5 densitometry

The autoradiographs were analysed using GS 670 densitometer from BioRad (Hecules, CA, USA). The software package used was Molecular Analysts Software version 2.1, Macintosh Software for BioRad's Image Analysis Systems.

The amount of RNA in each band is assumed proportional to the intensity of the bands. To calculate the relative amount of RNA in each band, a probe designed to 18S ribosomal RNA (2.6.2) was hybridised to the samples. The intensity of each band was measured and the background level, averaged from 10 background readings, subtracted from the value for each band. Values so calculated represent the relative levels of RNA in each sample.

Samples were then probed for the sequence of interest (the target sequence). Densitometric values for the amount of target RNA per band were then divided by the value obtained from the 18S rRNA band for that particular sample. These values represent the amount of target sequence per sample, after correction for differing amounts of sample in each lane.

corrected target RNA = target RNA / 18S rRNA

2.14.6 pre-flashing film

Autoradiography film was pre-flashed using a Sensitize™ PreFlash Unit RPN 2051 (Amersham). A 2 x 5cm slot was cut in a 50 x 50cm piece of card. Increments of 2.5cm were marked on a clamp stand, between 65-90cm from the darkroom benchtop. The Sensitize Unit was clamped to the stand at 65cm from the benchtop, upon which was placed a piece of Hyperfim MP (Amersham) covered by the prepared card. In this way, when the flash unit was fired, only the film beneath the 2 x 5 cm slot in the card was exposed. The card was moved, and the procedure repeated with the flash unit placed at increasing height increments of 2.5cm, exposing a new section of film at each height.

The optimum film-to-flash distance is that which produces an increase in OD_{540nm} = 0.1-0.2 on the developed film above that of the unexposed film.

Therefore, the film, exposed as described above, was developed and each exposure "window" measured spectrophotometrically. An unexposed section of film was used to zero the spectrophotometer at 540nm. The height at which the exposed film is 0.15 OD_{540nm} units above the OD_{540nm} of unexposed film is noted. When film is pre-flashed to sensitise it before use, the flash unit is placed at this distance from the film. Callibration was repeated for each new batch of film.

2.14.9 preparation of pronase

Dissolve pronase to 20mg/ml in 10mM Tris-HCl , pH7.5, 10mM NaCl. Self-digest to remove DNAse and RNAse at 37°C for 1 hour. Store at -20°C. Use at 37°C.

CHAPTER 3: METALLOTHIONEIN TRANSCRIPTS of *MONODELPHIS DOMESTICA* and *TACHYGLOSSUS ACULEATUS*

3.1 Introduction

Metallothionein has not previously been reported in a marsupial species. The ubiquity of the protein's expression throughout the vertebrates prompts us to anticipate its occurrence in this taxon. The eutherian mammals express a complex multigene family of metallothionein isoforms. The birds, on the other hand, express a very simple gene family of one, or rarely two, members. The marsupials diverged independently from the mammalian lineage after the mammal and bird lineages separated. This is roughly coincident with the amplification of the *MT* locus. Investigation of marsupial metallothionein, its structure compared to both eutherian and bird metallothioneins, and the extent of any amplification at the marsupial *MT* locus, may allow an estimation of the point in vertebrate history at which the *MT* genes underwent extensive duplication. This, in turn, may clarify the circumstances which instigated this series of duplications. Isolation of a marsupial metallothionein gene product from the South American opossum, *M. domestica*, is therefore undertaken here as the initial step in a clarification of the evolution of the mammalian *MT* gene family. The opossum, *M. domestica*, was chosen for several reasons:

1. a captive breeding colony is maintained at the University of Tasmania, under the direction of Prof. Norman Saunders. Consequently, tissues and expertise are readily available. Indigenous marsupial species in Australia, however, are largely protected by conservation edicts, with consequent prohibitive constraints on access.
2. several factors make the opossum a useful model animal for the study of metallothionein in the developing marsupial. Opossum young are born at an extremely early developmental stage, even for a marsupial. Because *M. domestica* is a pouchless marsupial, the young are more experimentally accessible than are the pouch-young of macropods etc. Furthermore, the

postnatal environment of the opossum can be more accurately compared with that of the eutherian mammal, than can the pouch environment of other marsupials.

3. the opossum is a member of the taxon presumed to be the oldest group of the mammalian subclass Marsupialia (Reig *et al.*, 1987) and therefore constitutes an ideal representative of the marsupials in phylogenetic analysis.
4. because the opossum serves as a model system for several branches of research (eg. Nicholls & Saunders, 1996; Kusewitt & Ley, 1996; Szymanski *et al.*, 1998; Aveskogh & Hellman, 1998; Xie *et al.*, 1998), data available for this species is accumulating more rapidly than for other marsupial species. The physiology of the opossum is therefore more comprehensively understood than is the physiology of other marsupials.

No metallothionein has been previously identified in any monotreme species. The monotremes fill an extremely interesting phylogenetic niche, and have been nominated an "evolutionary relic" because of their apparent low rates of morphological, physiological and molecular evolution (Shaw *et al.*, 1993; Kitsch *et al.*, 1997). They too diverged from the main mammalian line just before or after the multiple duplication of the eutherian *MT* locus. An investigation of metallothioneins in this group has, therefore, been undertaken to further refine the evolutionary history of the gene and to extend the accretion of sequence data for phylogenetic analysis.

3.2 RESULTS

3.2.1 Cloning metallothionein cDNA from *M. domestica*

3.2.1.1 RT-PCR of opossum metallothionein fragment: cloning and sequencing

Total RNA was extracted from the liver of an LPS-treated opossum. RT-PCR was performed with PCR primers designed to conserved regions of the mouse *MT1* coding sequence (Fig. 3.1) at an annealing temperature of 40°C, using mouse liver and sheep liver RNA as positive controls (Fig. 3.2). A band

Figure 3.1 Mouse metallothionein cDNA sequences with PCR priming sites

Conserved amino acids are given above the sequence in italised IUPAC-IUB code.

```
mMT1   CTC CTC ACT TAC TCC GTA GCT CCA GCT TCA CCA GAT CTA GGA
mMT2   AAC CGA TCT CTC GTC GAT CTT CAA CCG CCG CCT CCA CTC GCC
mMT3   CTT GCC GGG AGG AAC CAA GCT ACG GCG GCT GCT GGA CTG GAT
mMT4   CTG CCT CAG CCT CCC TTT CTT AGC TGC TCA CAC ACC TGG ACC

         M   D   P           C       C                   G       C
mMT1   ATG GAC CCC AAC --- TGC TCC TGC TCC ACC GGC GGC TCC TGC
mMT2   ATG GAC CCC AAC --- TGC TCC TGT GCC TCC GAT GGA TCC TGC
mMT3   ATG GAC CCT GAG ACC TGC CCC TGT CCT ACT GGT GGT TCC TGC
mMT4   ATG GAC CCT GGG GAA TGC ACG TGT ATG TCT GGA GGG ATC TGC

           C               C       C           C       C
mMT1   ACT TGC ACC AGC TCC TGC GCC TGC AAG AAC TGC AAG TGC ACC
mMT2   TCC TGC GCT GGC GCC TGC AAA TGC AAA CAA TGC AAA TGT ACT
mMT3   ACC TGC TCG GAC AAA TGC AAG TGC AAG GGC TGC AAA TGC ACG
mMT4   ATC TGC GGA GAT AAT TGC AAA TGC ACA ACC TGC AGC TGT AAA

           C       K   S   C   C       C   C   P       G   C
mMT1   TCC TGC AAG AAG AGC TGC TGC TCC TGC TGT CCC GTG GGC TGC
mMT2   TCC TGC AAG AAA AGC TGC TGC TCC TGC TGC CCC GTG GGC TGT
mMT3   AAC TGC AAG AAG AGC TGC TGC TCC TGC TGC CCT GCC GGA TGT
mMT4   ACC TGT CGT AAA AGC TGC TGT CCC TGC TGT CCC CCA GGC TGT

           K   C               C       C   K
mMT1   TCC AAA TGT GCC CAG GGC TGT GTC TGC AAA GGC --- --- ---
mMT2   GCG AAG TGC TCC CAG GGC TGC ATC TGC AAA GAG --- --- ---
mMT3   GAG AAG TGT GCC AAG GAC TGT GTG TGC AAA GGT GAA GAG GGG
mMT4   GCC AAG TGT GCC CGG GGC TGC ATC TGC AAA GGG --- --- ---

                               K   C       C   C       stop
mMT1   --- --- --- GCC GCG GAC AAG TGC ACG TGC TGT GCC TGA TGT
mMT2   --- --- --- GCT TCC GAC AAC TGC AGC TGC TGT GCC TGA AGG
mMT3   GCC AAG GCA GAG GCC GAG AAA TGC AGC TGC TGC CAG TGA GGA
mMT4   --- --- --- GGT TCA GAC AAG TGC AGC TGC TGT CCC TGA AAC

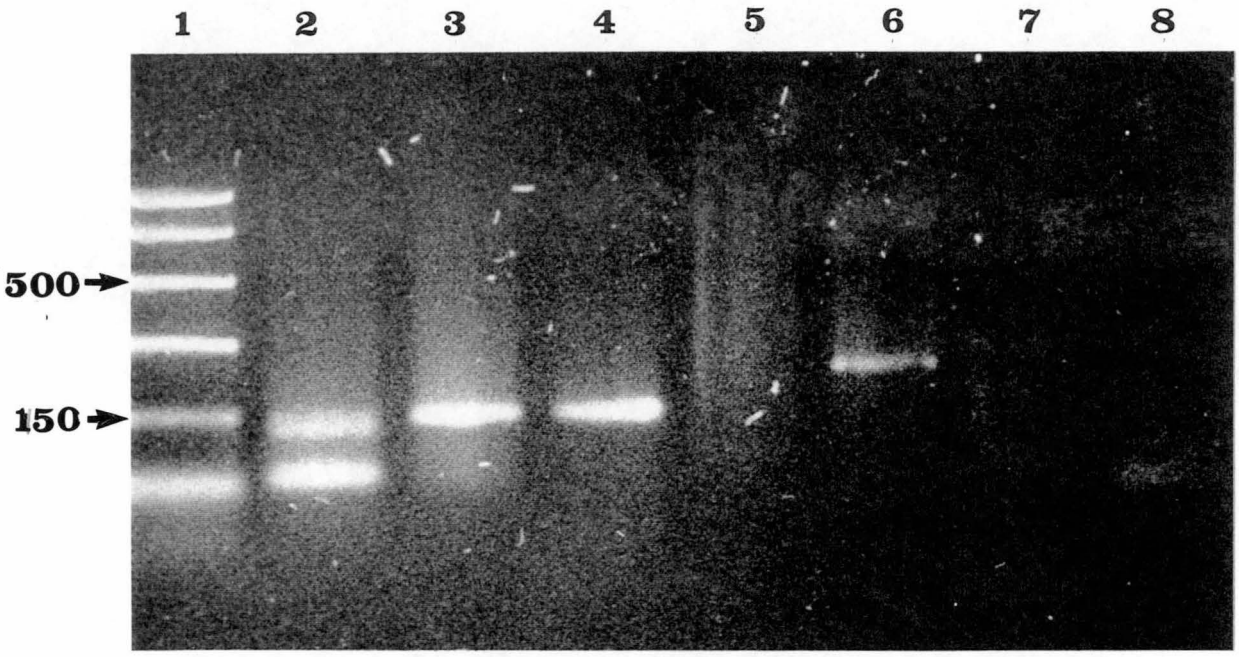
mMT1   GAC GAA CAG CGC TGC CAC CAC GTG TAA ATA GTA TCG GGA CCA
mMT2   GGG GCG GAG GGG TCC CCA CAT CTG TGT AAA TAG ACC
mMT3   CCC AGA CCC TCC CAC ACA GCC TAT GTA AAT AGT GCT GGG TGT
mMT4   CCA CCT ATG GCA GCG GGA GAG ATC CTG GGA AGT GAC TAC ACA

mMT1   ACC CAG CGT CTT CCT ATA
mMT2
mMT3   CCC TGG TGG GGC ACA AC
mMT4   GTA CCT GAG TCA AGG GAT
```

MT1 forward:
MT1 reverse:
Degenerate MT3 forward:
Degenerate MT3 reverse:
MT4 forward:

MT2 forward:
MT2 reverse:
MT3*Pst*I (forward):
MT3*Sac*I (reverse):
MT4 reverse:

Figure 3.2 RT-PCR of opossum, mouse and sheep liver RNA using conserved primers.



1. Markers -1kb, 750bp, 500bp, 300bp, 150bp, 50bp.
2. Opossum liver RNA with conserved primers
3. Mouse liver RNA with conserved primers
4. Sheep liver RNA with conserved primers
5. Opossum liver RNA with mMT2 primers
6. Mouse liver RNA with mMT2 primers
7. Sheep liver RNA with mMT2 primers
8. no DNA control

The second band amplified from opossum RNA (lane 2) and running at 50 bp, was shown to be single-stranded PCR product.

was observed at the expected size (approx. 150 bp) and was cloned into the *Sma*1 site of the pUC19 vector and selected by screening with purified PCR product, which was radiolabelled with α-^{32}P-dCTP using T7 polymerase. Plasmids were isolated and the nucleotide sequences established for the two longest and the two shortest cloned fragments by the Sanger dideoxy method. Three of the clones gave rise to apparent metallothionein sequence. These sequences were translated using the "translate" program of the GCG software package into primary protein sequence recognizable as class I metallothionein. The sequence of the longest fragment, which is referred to hereunder as "MdMT", is given in Fig.3.3 together with the corresponding primary amino acid sequence. The sequences of the other two clones were identical to that in Fig. 3.3, albeit shorter.

Figure 3.3

GGA GAC TCT TGC AAA TGC AAA TCA TGC TCC TGT ACC TCC TGT AAG

G D S C K C K S C S C T S C K

AAA AGC TGC TGC TCC TGC TGC CCA GTG GGA TGT GCC AAG TGT GCC

K S C C S C C P V G C A K C A

Nucleotide and derived amino acid sequences of cloned PCR fragment isolated from *Monodelphis domestica* liver cDNA using primers to regions of mouse *MT1*. The PCR product was prepared for blunt-end ligation with mung bean nuclease, and consequently the cloned fragment is shorter than the amplified sequence.

3.2.1.2 Construction and screening of *Monodelphis* brain and liver cDNA libraries isolates four MT sequences

RNA was extracted from the brains and liver of two adult *M. domestica* by a method modified from Chomczynski and Sacchi (1987). A cDNA library was constructed for each tissue in the ZAP Express™ pBK-CMV vector, as described (section 2.10).

Packaged libraries were plated with XL1-Blue MRF' cells onto IPTG/x-gal NZY agarose and white recombinant plaques counted. The libraries were amplified once and titres measured using serial dilutions with host cells.

* brain library recombinants = 266,000, brain library titre = 7 x 10^9 pfu/ml
* liver library recombinants = 172,500, liver library titre = 1 x 10^7 pfu/ml

Subsets of both brain (56,000 pfu) and liver (40,000 pfu) libraries were screened with radiolabelled MdMT fragment at low stringency (37°C in 50% formamide). Positives conservatively ascertained in the primary screen:

brain = 18

liver = 49

Of these, 8 plaques from the brain library and 7 plaques from the liver library were taken through to secondary and tertiary screens. Secondary and tertiary screens were performed at 60°C, in 50% formamide buffer (washed in 0.2 x SSC; 1% SDS at 50°C) with no loss of positives. Tertiary screen was probed with both mouse MT3 (mMT3) cDNA and MdMT cDNA at this stringency. All clones responded as positives for the *Monodelphis* MdMT probe. No positives were recorded for the mMT3 probe, after washing in 0.2 x SSC; 1% SDS at room temperature.

Phagemids were excised from ZAP Express vectors for all positive phage (8 brain, 7 liver), using ExAssist helper phage and XL1-Blue MRF' according to manufacturer's protocol. Phagemids were plated with XLOLR host cells onto LB-kanamycin plates. Single colonies were incubated in LB broth, and phagemids extracted with alkaline lysis, and restricted with *Sac*I and *Xba*I. The restriction products were separated on 1% agarose before Southern blotting onto nylon membrane. Membranes were probed with radiolabelled MdMT fragment. All phagemids were positive for metallothionein and were sequenced using the ABI PRISM™ Dye Primer Cycle Sequencing method.

Four distinct sequences were isolated, each as several individual clones. The amino acid sequences derived from these clones conform to the criteria for class I metallothioneins. The cDNA sequences are given in Fig. 3.4 and the sequencing strategy for each is given in Fig. 3.5. The prefixes B and L indicate

B3 CCTCCTTCTGCTGTGAGACCTGCATCCCGACATCTATCTGCCAGCC*ATG*GACCCCAACTGTGACTGCGTGAGCGGAAGTTCTTGCACC
L2 GAGAGGTTAACGTCTCCCTGCCAGCC*ATG*GACCCCGACTGTGACTGCGTGAGCGGTGGCTCTTGCACC
L3 CTTGCTCGACCCGCAGAGAGAACTCCAGACTTCCTAGTT*ATG*GACCCCAACTGTAACTGCGCCAACGGTGGCTCCTGCACC
B1 CCATCCTTCTCCTGTGAGCTCTGCGTGCCTGACGTGTACCTGCCAGCC*ATG*GACCCCAAATGCAACTGCGAGAGTGGAGGTTCTTGCACC

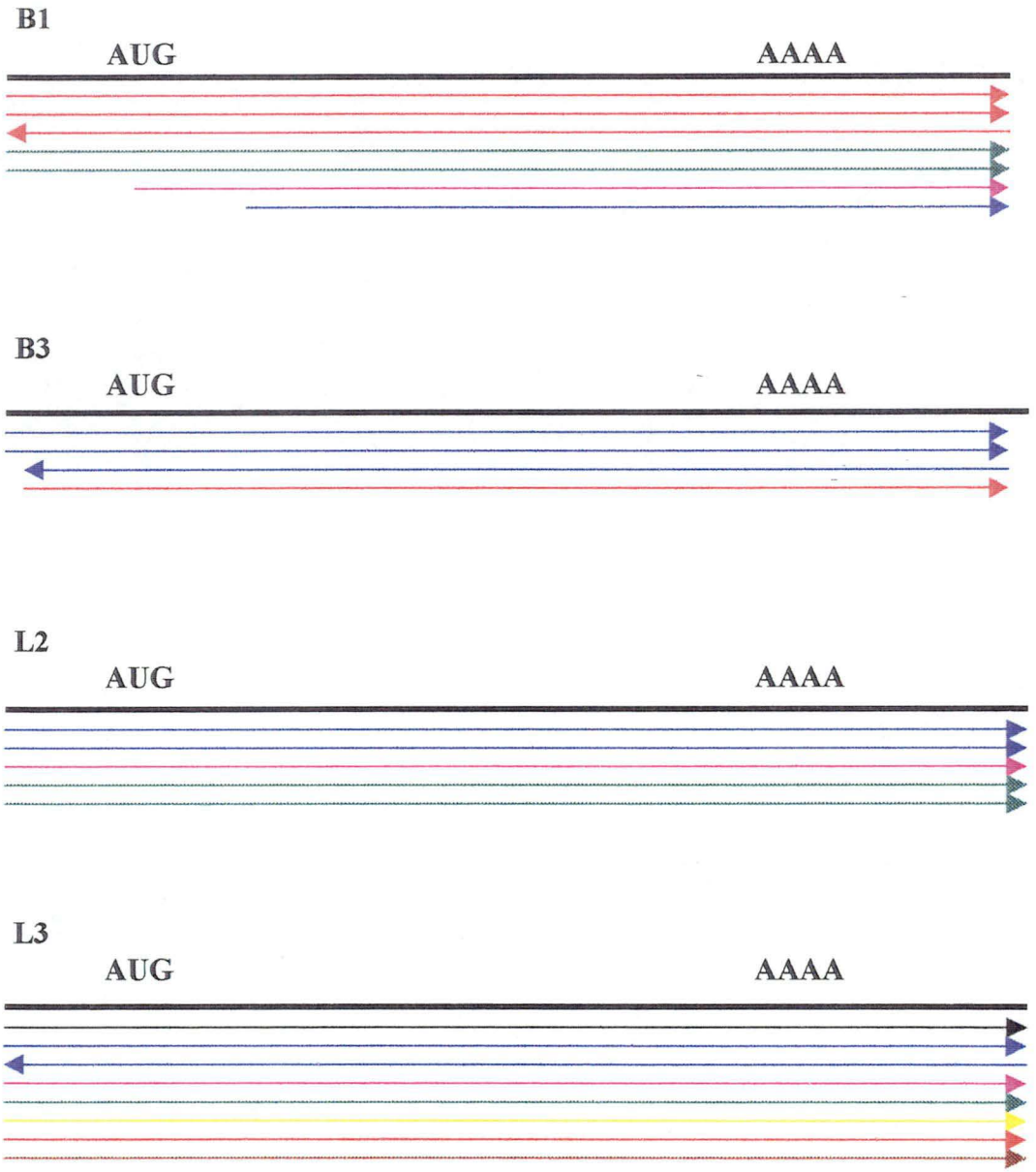
B3 TGTGCAGGCTCCTGCAAGTGTAAATCCTGCCGATGTACCTCCTGCAAGAAAAGCTGCTGCTCCTGCTGCCCAGCGGGATGTGCCAAGTGTGCCCAGGGCTGTG
L2 TGTGCAAACTCCTGCAAATGTAAATCTTGTCAGTGTACCTCCTGCAAGAAAAGCTGCTGCTCCTGTTGCCCAGTGGGATGTGCCAAGTGTGCTCAGGGCTGTG
L3 TGCGGAGACTCTTGCAAATGCAAATCATGCTCCTGTACCTCCTGTAAGAAAAGCTGCTGCTCCTGCTGCCCAGTGGGATGTGCCAAGTGTGCGCAGGGCTGTG
B1 TGTGCAGGCTCCTGCAAATGTAAATCCTGCCGCTGCACCTCCTGCAAAAAAAGCTGCTGCTCCTGCTGCCCAGTGGGATGTGCCAAGTGTGCCCAGGGCTGTG

B3 TCTGCAAAGCCCCCCAGACTGAGAGTTGCAGCTGCTGCCAC*TGA*TGCTCCTCTCTCCCATGACTGTAAATAGAAGAATTTGTACAGACTTGACTTTTCATACA
L2 TCTGCAAAGCCCCCCAGACTGAGAGTTGCAGCTGTTGCCAC*TGA*TGCTCC**CATTTTTCTTTGCCTGTA**AATAGAAGTTGTACAGACCTGAATTATCCTATACC
L3 TCTGCAAAGCCCCCCAGACTGAGAGTTGCAGCTGTTGCCAC*TGA*TGTTCC**CACCTCCTCATTCCTGTA**GATAGAAGATGCCATCTGTTTTTGTGCACCATGAA
B1 TCTGCAAAGCCCCCCAGACTGAGAGTTGCAGCTGCTGCCAG*TGA*TGTTCCTGTCTTCTTGTGCCTGTAGATAGAGGAATTTGTACAGACCTGATTTTTTTATA

B3 CTTTGGTCATATTTTTCTATTTTTTTTCTATAAATGTGTGAT**TCAATCCACAGGCATTTA**TTAACCACTTAGTATGCC*aataaa*
L2 AAAGATATGTATTCTACTGTTTTTCAATGAAGCATG*aataaa*
L3 TGTTTTTCTACTGTTTTTCCTATGAAATGTATAAATGAA*aataaa*
B1 CAC**TGAGTTGTGTTTTCTAA**TCTTTTTTCCACTATGAAATGAA*aataaa*

Figure 3.4 Aligned sequences of four *Monodelphis* metallothionein cDNAs. Isoform-specific oligonucleotide probe sites are underlined boldface. Putative translation start sites and termination codons are italicised and underlined; putative polyadenylation signals are italicised lower case. Sequences have been truncated at polyadenylation signal site.

Figure 3.5: Sequencing strategy for four *Monodelphis* metallothionein cDNA molecules. Arrows of different colours indicate sequence derived from multiple independent clones, each of which had identical sequence.



that the clone was originally isolated from the brain or liver library, respectively. However, after several rounds of screening, each sequence was isolated from both cDNA libraries. The transcript L3 can be seen to correspond to a full-length coding region of the PCR fragment MdMT (Fig. 3.3).

3.2.1.3 Design and specificity of four cDNA-specific probes

Sequences for the *Monodelphis* metallothionein cDNAs were aligned. Unique 17 or 18 base pair sequences in the 3' untranslated regions of each were established (Fig. 3.4). The reversed complementary sequences to these sites (section 2.7.2) were analysed with the computer program Oligo 5.0 to preclude hairpin loops or interoligonucleotide duplices, and to confirm theoretical cDNA-specificity. Oligonucleotides were manufactured by Bresatec and specificity demonstrated by probing each of the cloned *Monodelphis* metallothionein sequences with each of the oligonucleotide probes (Fig. 3.6)

3.2.1.4 Differential screening of brain and liver libraries isolates a fifth MT sequence

To establish if further MT cDNAs occur in either the brain or liver of *M. domestica*, differential screens of both libraries were performed. 150,000 recombinant phage of the brain library and 60,000 recombinant phage of the liver library were plated and duplicate phagelifts taken from each plate. One of each duplicate filter was screened with a combination of all four sequence-specific probes in 40% formamide buffer at room temperature. The other duplicate filter was hybridised with the generalised MdMT cDNA in 50% formamide buffer at 56°C. By overlaying autoradiographs of each screen, phage positive for the generalised probe but negative for the sequence-specific probes were identified and presumed to be possible new transcripts.

Eight possible new clones were isolated from the brain library and processed as described. Six possible new clones were isolated from the liver library and sequenced. Of these fourteen sequences, three represented a heretofore undiscovered sequence which occurred in both libraries. This

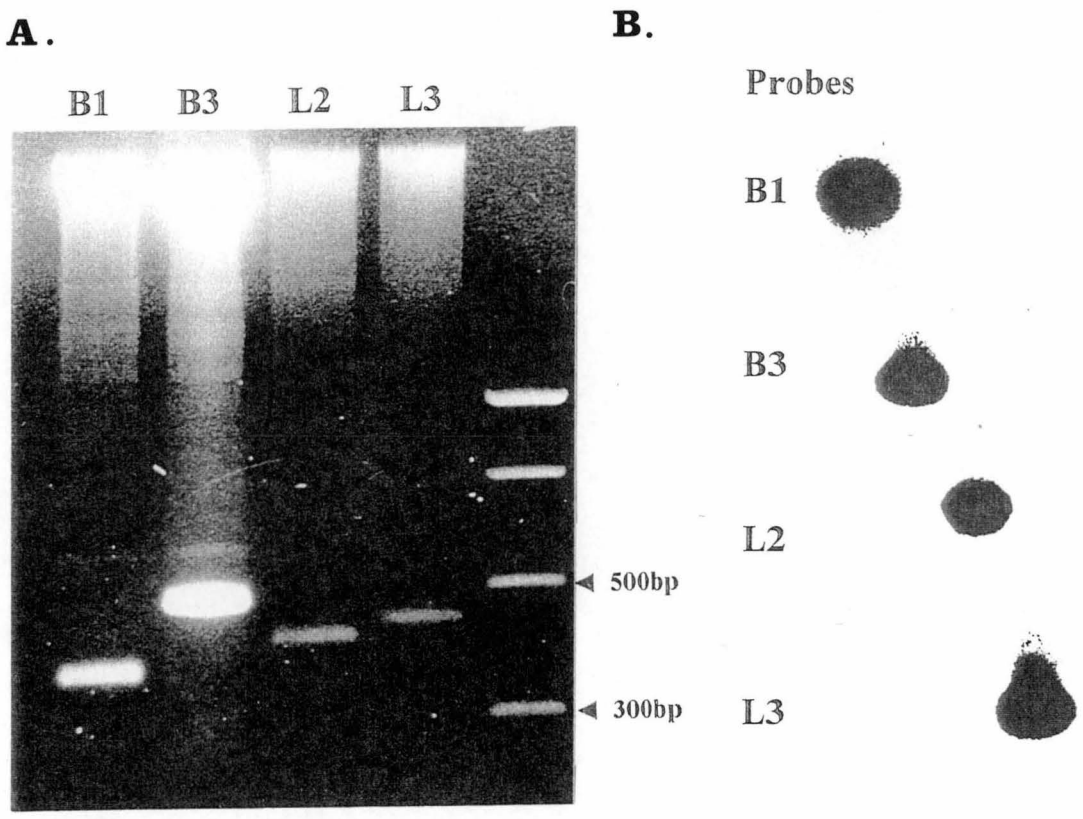


Figure 3.6

A. Plasmids containing each cloned *Monodelphis* metallothionein cDNA were doubly restricted with *Xba*I and *Sac*I. Restriction products were electrophoresed on a 1% agarose/TBE gel with ethidium bromide, and visualised under transillumination. Gel was blotted onto nylon membrane.

B. cDNA-specific probes were labelled with ^{32}P-α-dCTP using terminal transferase. Each was used, in turn, to probe the filter at a hybridization temperature of 20°C in 40% formamide buffer. Blots were washed in 0.1 x SSC, 1% SDS at 37°C for 20 min. and exposed to pre-flashed film at -80°C with intensifying screens for 1-3 hours.

transcript is designated MTx5, and its sequence given in Fig. 3.7a. The sequencing strategy for these clones is given in Fig. 3.7b. Of the remaining sequences, four were the previously described B3 sequence, four the previously described B1 sequence, one the previously described L2 sequence and two could not be identified as metallothioneins.

3.2.1.5 Derived amino acid sequences of MT cDNAs

Derived amino acid sequences of the five metallothioneins of *Monodelphis* are given in Fig. 3.8, together with the amino acid sequence of the four isoforms of mouse. (Echidna sequences will be discussed below).

Distinctive characteristics can be identified for the five metallothioneins of the opossum. Each isoform consists of 62 amino acids. A proline is conserved at residue 52a at the site of the carboxyterminal domain insertion in mammalian MT3.

MTs 1 and 2 of eutherian mammals almost invariably have alanine as carboxyterminal residue (with the exception of human MT1f). Derived *Monodelphis* sequences B1 and x5 have a terminal glutamine in common with mammalian MT3 and fish MTs. Sequences B3, L2 and L3 have a carboxyterminal histidine in common with bird MTs (Wei & Andrews, 1988; Fernando & Andrews, 1989; Lin *et al.*, 1990a, 1990b; Lin & Huang, 1990; Shartzer *et al.*, 1993). Histidine is rarely found in metallothionein molecules, except in this instance as the carboxyterminal residue in bird proteins. *Monodelphis* putative isoform x5 also has an histidine residue at site 25 (Fig. 3.8). Histidine within the body of the protein is highly unusual and the only other documented occurrence is in the metallothionein of *Xenopus laevis* at residue 50 (*X. laevis* numbering) (Muller *et al.*, 1993; Saint-Jacques & Seguin, 1993).

The predicted *Monodelphis* MT isoforms show remarkable primary sequence conservation within the α-domain (Fig. 3.8, sites 32-61) of the molecule. Apart from the dichotomy in carboxyterminal residues, variation occurs only at residue 39, which is either an alanine (B3) or a valine (B1, L2, L3 and x5), conservative alternatives that are prevalent throughout the vertebrate

Figure 3.7a Nucleotide sequence and derived amino acid sequence for the fifth *Monodelphis* metallothionein cDNA, MdMTx5. Putative translation start site and termination codon are boldface and underlined: predicted polyadenylation signal is italicised lower case. Derived amino acid sequence is given below in IUPAC-IUB single letter code. * indicates probe site for cDNA-specific oligonucleotide.

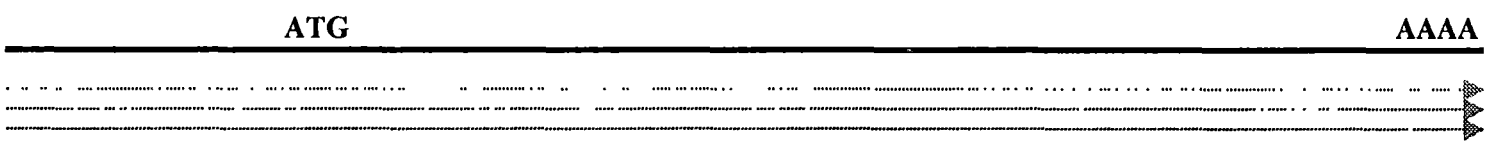
```
CAACCTTCCAGCCCATCCTTTTCCTCTGAGACTTGAGCACCTGCATCTACCGGCCAGCCATGGACCCCAATTGTGACTGTGGGAATGGTGCCTCTTGTACC
                                                          M  D  P  N  C  D  C  G  N  G  A  S  C  T

TGTGCAGGCTCCTGCAAATGTGCATCCTGTCACTGTACTTCCTGCAAGAAAAGCTGCTGCTCCTGTTGCCCAGTGGGATGTGCCAAGTGTGCCCAGGGCTGT
 C  A  G  S  C  K  C  A  S  C  H  C  T  S  C  K  K  S  C  C  S  C  C  P  V  G  C  A  K  C  A  Q  G  C

GTCTGCAAAGCCCCCCAGACTGAGAGTTGTAGCTGCTGTCAGTGATGGACCTGCCCCCCTGTACTTGTAAATAGAGGAATTTGTTCAGATGTGGCTTTTTAT
 V  C  K  A  P  Q  T  E  S  C  S  C  C  Q                                                  ********************

ACACTGTAGATGTACTTTCTGCTTTGTTTCCATGAAATTTGTGAATGAAaataaa
***********
```

Figure 3.7b Sequencing strategy for the fifth *Monodelphis* metallothionein cDNA, MdMTx5. Each arrow indicates sequence derived from an independent clone.



```
                                  1111111111222222222233333333334444444444555      555555566
                          123a456789012345678901234567890123456789012345678901abcdef345678901
opossum B1        MDP KCNCESGGSCTCAGSCKCKSCRCTSCKKSCCSCCPVGCAKCAQGCVCKAP      QTESCSCCQ
opossum B3        MDP NCDCVSGSSCTCAGSCKCKSCRCTSCKKSCCSCCPAGCAKCAQGCVCKAP      QTESCSCCH
opossum L2        MDP DCDCVSGGSCTCANSCKCKSCQCTSCKKSCCSCCPVGCAKCAQGCVCKAP      QTESCSCCH
opossum L3        MDP NCNCANGGSCTCGDSCKCKSCSCTSCKKSCCSCCPVGCAKCAQGCVCKAP      QTESCSCCH
opossum x5        MDP NCDCGNGASCTCAGSCKCASCHCTSCKKSCCSCCPVGCAKCAQGCVCKAP      QTESCSCCQ

echidna 2         MDPQDCGCATGGSCACATSCKCKDCRCTSCKKSCCSCCPVGCAKCAQGCVCKEP      QTDKCSCCQ
echidna 5         MDPQNCGCATGGSCTCAGSCKCKDCRCTSCKKSCCSCCPVGCAKCAQGCVCKEP      QTDKCSCCQ

mouse 1           MDP NCSCSTGGSCTCTSSCACKNCKCTSCKKSCCSCCPVGCSKCAQGCVCKG       AADKCTCCA
mouse 2           MDP NCSCASDGSCSCAGACKCKQCKCTSCKKSCCSCCPVGCAKCSQGCICKQ       ASDKCSCCA
mouse 3           MDPETCPCPTGGSCTCSDKCKCKGCKCTNCKKSCCSCCPAGCEKCAKDCVCKGEEGAKAEAEKCSCCQ
mouse 4           MDPGECTCMSGGICICGDNCKCTTCNCKTCRKSCCPCCPPGCAKCARGCICKG       GSDKCSCCP
```

Figure 3.8

Primary amino acid sequences of the putative metallothionein isoforms of *M. domestica* (opossum), *T. aculeatus* (echidna) and of mouse. Sequence breaks accommodate inserts in some sequences. Sequence sites are numbered in red, relative to mouse MT1. Insertion sites are represented by lower case letters.

metallothioneins. This constitutes a degree of similarity between α domains of 96.8-98.4%. In the β-domains (residues 1-29) of the *Monodelphis* MT isoforms, on the other hand, there are eight positions at which amino acids differ, making the β-domains of the various isoforms between 77-87% similar (Table 3.1). Conservation of the α-domain may be attributable to either selection pressure or, if the conservation is reflected in sequence conservation at the DNA level, gene conversion. Overall similarity between the metallothionein molecules ranges from approximately 87-90%.

Table 3.1: % similarity between predicted protein sequences of metallothionein isoforms of *M. domestica* - combined domains (above the diagonal) and β-domain (below the diagonal).

	B1	B3	L2	L3	X5
B1	-	88.7	90.3	88.7	90.3
B3	87.0	-	90.3	87.1	88.7
L2	83.8	87.1	-	88.7	87.1
L3	80.6	77.4	77.4	-	87.1
X5	77.4	83.8	77.4	77.4	-

3.2.1.6 Classification of the Predicted MT isoforms of *Monodelphis*

3.2.1.6.1 Primary protein structure of predicted *Monodelphis* MTs

Predicted *Monodelphis* metallothioneins have several features in common with eutherian MTs 1 and 2. There is no insert in the N-terminal domain corresponding to that found in eutherian MT3 and MT4. A serine occurs in each *Monodelphis* isoform at the site corresponding to Ser28 (numbered as in Fig. 3.8) conserved in eutherian MTs 1and 2. *Monodelphis* metallothioneins also share residue Gln46 conserved in the eutherian metallothioneins 1 and 2. Conservation of these amino acids does not extend to MTs 3 and 4. No acidic residue occurs at site 10 or 11 of any opossum MT polypeptide. By accepted criteria, these MTs cannot be classed as MT2.

Monodelphis MTs also share sequence characters with eutherian MT3. The single proline insertion in the α-domain of the five derived *Monodelphis* metallothioneins occurs at the site corresponding to the 4-6 residue insert in the α-domain of eutherian MT3. Furthermore, *Monodelphis* B1 and x5 isoforms have a glutamine as carboxyterminal residue, as do eutherian MT3s.

There is little similarity between the metallothioneins of *Monodelphis* and the two eutherian MT4 proteins, beyond the characters common to all metallothioneins.

3.2.1.6.2 Apothionein charges

Eutherian MTs 1 and 2 were originally distinguished on the basis of their chromatographic separability by charge. Subsequently, this characteristic has been reinterpreted as the occurrence or not of an acidic residue at site 10 or 11 of the polypeptide. To test the validity of using molecular charge as a basis for classification of the metallothioneins, the theoretical charges were calculated for the various vertebrate (metal-free) apothioneins using the Protean program of the DNAStar package.These are listed in Table 3.2.

Table 3.2 Calculated charges on apothionein molecules of different species, using the Protean program of the DNAStar package. Sequences are taken from Appendix 2. Asterix (*) identify charges that are atypical for that isoform or taxonomic group.

	MT1	**MT2**	**MT3**	**MT4**
human 1a	+4.29	+3.29	-3.69	+4.29
1b	+4.29			
1e	+4.29			
1f	+3.29*			
1g	+4.29			
1i	+4.29			
1k	+4.29			
1x	+4.29			
monkeyA	+3.29*			
monkeyB		+3.29		
mouse	+4.29	+3.29	+1.3	+3.29
rat	+4.29	+3.29	+2.3	

rabbit	+4.29	+3.29 2a		
		+3.29 2b		
		+3.29 2c		
		+4.29* 2e		
		+4.29* 2f		
sheep 1a	+4.29	+3.29	-3.6	
1b	+4.29			
1c	+4.29			
horse 1a	+4.29		-3.69	
1l	+4.29			
1r	+5.29*			
1b	+4.29			
bovine	+4.29	+3.29	-3.69	
pig	+4.29			
dolphin		+4.29*		

opossumB1	+4.29
opossumB3	+3.49
opossumL2	+1.46
opossumL3	+2.46
opossumx5	+1.46
echidnaA	+2.29
echidnaB	+3.29
muscovy	+4.46
duck	+4.46
chicken	+4.46
pigeon1	+4.32
pigeon2	+3.46
xenopus	+2.49
flounder	+2.29
plaice	+2.29
troutA	+2.29
troutB	+2.29
bream	+2.29
cod	+1.29*
pike	+2.29
loach	+2.29
goldfish	+3.29*
perch	+2.29
icefishA	+2.29
icefishB	+2.29
icefishC	+2.29

With minimal exceptions (* in Table 3.2), eutherian MT1s have a calculated charge of +4.29, while MT2s have a calculated charge of +3.29. (Dolphin MT2 has been misclassified and should be designated an MT1 on the criterion of non-acidic residues at sites 10 and 11). From these data, therefore, the MT1 and MT2 isoforms of eutherian mammals can be classified in accord with the theoretical charge on the protein. All eutherian mammals have a representative molecule with a charge of +4.29 and another with a charge of +3.29. With the exception of horse MT1r, no MT1 or 2 molecule has a charge other than +4.29 or +3.29. Eutherian MT3s, on the other hand, display a range of charges from -3.69 (human, cow, horse, sheep) to +2.3 (rat). Only two MT4 isoforms are presently described: the human isoform with a calculated charge of +4.29 , the mouse +3.29. The metallothionein molecules of other vertebrate species have charge in approximate accord with taxonomic groupings (eg fish +2.29).

The predicted metallothionein molecules of *Monodelphis* range in charge from +1.46 to +4.29. Although isoform B1 has a calculated charge corresponding to that of eutherian MT1 proteins, the opossum metallothioneins as a group cannot easily be fitted into the classification of mammalian metallothioneins on the basis of their theoretical charge.

3.2.1.6.3 Tissue distribution

Eutherian metallothioneins can be defined largely on the pattern of their distribution within the various tissues of the organism. MTs 1 and 2 are expressed in many different tissues. MT3 is restricted mainly to the CNS (Uchida *et al.*, 1991), some reproductive organs, heart, stomach and tongue (Moffatt & Seguin, 1998), but does not occur in the liver. MT4 mRNA has an even more restricted distribution to specialised squamous epithelia of highly localised regions (Quaife *et al.*, 1994).

Each of the metallothioneins from *M. domestica* described here was isolated from both brain and liver cDNA libraries. By the classification accepted

for eutherian mammals, none of these putative opossum isoforms could therefore be defined as an MT3 or MT4.

3.2.1.6.4 Summary

By considerations of primary structure, the predicted metallothioneins of *Monodelphis* presented here are an homogeneous group that have features in common with both eutherian MTs 1 and 2, and eutherian MT3. On the basis of tissue distribution however, no *Monodelphis* metallothionein can be likened to eutherian MT3 or MT4. The tight correlation between apothionein charge and isoform that can be demonstrated for the eutherian MTs 1 and 2 does not occur in the opossum MTs. Therefore, the opossum metallothioneins are not readily accommodated in the current classification of mammalian MTs.

3.2.2 Looking specifically for *Monodelphis MT3*

The suite of characters that distinguish mammalian MT3 from the other mammalian isoforms cannot be attributed to any of the predicted *Monodelphis* metallothioneins. Of the two insertions typical of MT3, one is completely absent from the opossum metallothioneins and the other is truncated. Furthermore, all metallothionein cDNAs described from *Monodelphis* occur in the liver. The tissue specificity characteristic of MT3 suggests functional specialization that cannot be postulated for the more widely expressed opossum metallothioneins. The question arises whether the opossum expresses a further metallothionein protein corresponding to the eutherian MT3. Sequence and expression properties of eutherian MT3 were used to investigate the existence of such an isoform.

3.2.2.1 RT-PCR with *MT3*-specific primers

RT-PCR was performed on RNA extracted from the brain of adult *Monodelphis*, using two different sets of PCR primers. Primers were designed to mouse *MT*, the forward primer extending over the 5' insertion sequences (Fig. 3.1). Both primers incorporate 5' restriction sites (lower case).

forward primer

mMT3*Pst*1: 5'-CCctgcagTGGATATGGACCCTGAGACC-3'

reverse primer

mMT3*Sac*1: 5'-CCgagctcGCTGCAYTTYTCNGCYTC-3'

Y = C or T N = any nucleotide

PCR was performed at low stringency (annealing temperature of 32°C) to specifically isolate the cDNA of an MT3 homologue. Mouse brain cDNA was used as a positive control. No band corresponding to *Monodelphis* MT3 was isolated (results not shown). The reaction was repeated using the degenerate MT3 primers designed by Palmiter *et al.* (1992), to the conserved regions of mouse MT3 mRNA (Fig. 3.1; section 2.8.4). Similarly, no band corresponding to *Monodelphis* MT3 was isolated (results not shown).

3.2.2.2 Northern analysis

Northern analysis was used to investigate evidence for an homologue of eutherian *MT3* in *Monodelphis*. A degenerate 21mer oligonucleotide was designed to a consensus sequence derived from documented mammalian *MT3* nucleotide sequences. The consensus includes the region coding for the CPCP amino acid motif that distinguishes the MT3 proteins and to which is attributed the *in vitro* neuron inhibitory action of human MT3 (Sewell *et al.*, 1995)(Fig. 3.9).

The oligonucleotide was used to probe total RNA from the brains and livers of echidna, opossum, rat, sheep and mouse at a hybridization temperature of 40°C in 40% formamide buffer, and washed in 0.1 x SSC at RT (Figs. 3.10a,b). (Rat liver RNA had degraded and is therefore not discussed in this analysis). Mouse brain, rat brain and sheep brain were positive for MT3 conserved sequence at this stringency; echidna and opossum brain RNAs were not. No positive signal was detected in any liver sample at this stringency. Mouse MT3 cDNA was used to probe this blot in 50% formamide at 37°C (washed in 0.1 x SSC, 1% SDS at room temperature) (Fig. 3.10c). No signal

occurs in either echidna or opossum brain RNA, although low level signal is discernible in the liver RNA of mouse and sheep.

Figure 3.9: Consensus sequence of known MT3 cDNAs

Conserved primary sequence in the β-domain of eutherian MT3s:

```
M   D   P   E   T   C   P   C   P   T   G
            *   *   *   *   *   *   *
```

Highly conserved corresponding nucleotide sequences:

```
human                GAG ACC TGC CCC TGC CCT TCT   #1.

pig                  ... ... ... ... ... ... A..   #2.

sheep                ... G.. ... ... ... ... A..   #3.

mouse                ... ... ... ... ..T ... A..   #4.

rat                  ... ... ... ... ..T ... A..   #5.

consensus:       5'-GAG ACC TGC CCC TGY CCT ACT-3'

reverse          5'-AGT AGG RCA GGG GCA GGT CTC-3'
complement

                     Tm  = 70°C
                     G+C = 66%

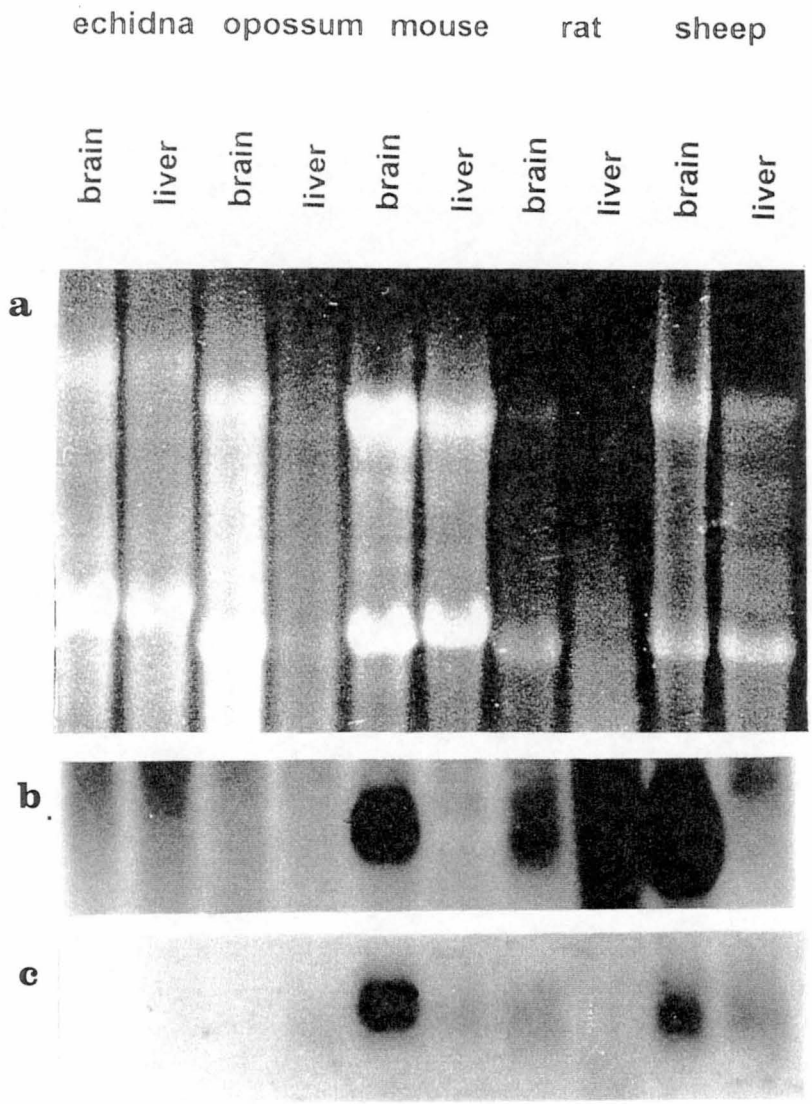
R = A or G
Y = C or T
```

#1. Palmiter *et al.*, 1992/ Tsuji *et al.*, 1992
#2. Chen *et al.*, 1996
#3. Holloway, 1996
#4. Palmiter *et al.*, 1992
#5. Kobayashi *et al.*, 1993

Together these results suggest that if an homologue to *MT3* occurs in the opossum brain, the sequence of the corresponding mRNA is sufficiently different from known *MT3*s to elude detection by these methods. Alternately, the gene may be expressed at such low concentration as to forestall detection by northern analysis.

The conserved degenerate *MT3*-specific oligonucleotide (Fig. 3.9) was also used to probe, at low stringency (40% formamide, 37°C hybridization;

Figure 3.10



A: Total RNA from the brains and livers of echidna, opossum, mouse, rat and sheep was separated on a 1% denaturing gel and blotted onto nylon membrane. RNA from the rat liver is degraded and is therefore not considered with the results.

B: The MT3 conserved oligonucleotide was labelled with ^{32}P-α-dCTP and hybridized in 40% formamide buffer (methods) at 40°C overnight. Membrane was washed to 37°C in 0.1 x SSC, 1% SDS for 40 min. and exposed to pre-flashed film at -80°C for 4 days with intensifying screens.

C: Mouse MT3 cDNA fragment was radiolabelled and hybridized in 50% formamide at 37°C overnight, washed in 0.1 x SSC, 1% SDS at room temperature and exposed to pre-flashed film at -80°C with intensifying screens overnight.

washed to 37°C in 0.2 x SSC, 1% SDS), RNA samples from various tissues of *Monodelphis*. After exposure to pre-flashed film at -80°C with intensifying screens for 3 weeks, there was no evidence of positive signal in any tissue (results not shown).

3.2.2.3 Screening the brain cDNA library for MT3

As described above, the *Monodelphis* brain library was screened with MdMT cDNA at low stringency (37°C in 50% formamide, washed to RT in 0.2 x SSC) and the tertiary screen of positives probed with mMT3 cDNA (described in section 2.7.1) at a stringency of 60°C in 50% formamide (washed in 0.2 x SSC, 1% SDS at 37°C). At this hybridization temperature, no positive plaques were scored.

When a further 80,000 recombinant phage from the brain library were screened with mMT3 cDNA at an hybridization temperature of 55°C, no positive plaques were scored.

750,00 pfu of the brain library were screened with sheep MT3 cDNA exons 1 and 2 (sMT3, described in section 2.7.1), because these exons code for the protein domain containing the highly conserved features that distinguish MT3 from other metallothioneins. At an hybridization temperature of 55°C in 50% formamide (washed to 37°C in 0.2 x SSC, 1% SDS), two positive plaques were isolated. These proved upon sequencing to be the metallothionein cDNA previously named L3. It was subsequently shown that at high stringency (65°C in 50% formamide, washed to 37°C in 0.2 x SSC, 1% SDS), the MdMT cDNA L3 hybridizes intensely with the sMT3 cDNA (results not shown).

3.2.2.4 Summary

These results suggest that there is sufficient homology between the various eutherian *MT3* nucleic acid sequences and the five MT cDNAs of *Monodelphis* to confound the isolation of an *MT3* homologue at moderate stringency. However, sequence conservation between this postulated homologue

and the eutherian *MT3*s is insufficient to allow detection of any such homologue with the MT3 cDNA probes at high stringency.

3.2.3 Looking specifically for *Monodelphis MT4*

The eutherian isoform MT4 mRNA has been shown to occur in neither the brain nor the liver. A principal site of MT4 mRNA expression is the tongue epithelium (Quaife *et al.*, 1994). Therefore, screens of either cDNA library are unlikely to isolate an homologue of *MT4* in *Monodelphis*. In the quest for an MT4 homologue in the opossum, the characteristic tissue specificity of *MT4* expression exploited by northern analysis.

3.2.3.1 Cloning mouse MT4 cDNA as a probe

Because PCR was unsuccessful in isolating an opossum homologue of eutherian *MT4*, mouse MT4 (mMT4) cDNA was cloned as a probe for opossum RNA. Eutherian MT4 transcript has been found in highly localised sites including the tongue epithelium, and mMT4 cDNA was used to explore such tissue-specificity in the opossum.

PCR primers were designed complementary to mMT4 cDNA non-coding sequences as shown in Fig.3.1. Restriction sites were added to the 5' ends of the primers (lower case) to facilitate cloning. *Xba*I and *Sac*I sites were chosen to avoid known restriction sites within the mMT4 cDNA molecule, for compatibility with the pUC19 vector and to avoid cross-dimerization of oligonucleotides.

forward primer

mMT4*Sac*I: 5'-CCgagctcCAGCCTCCCTTTCTTAGCTG-3'

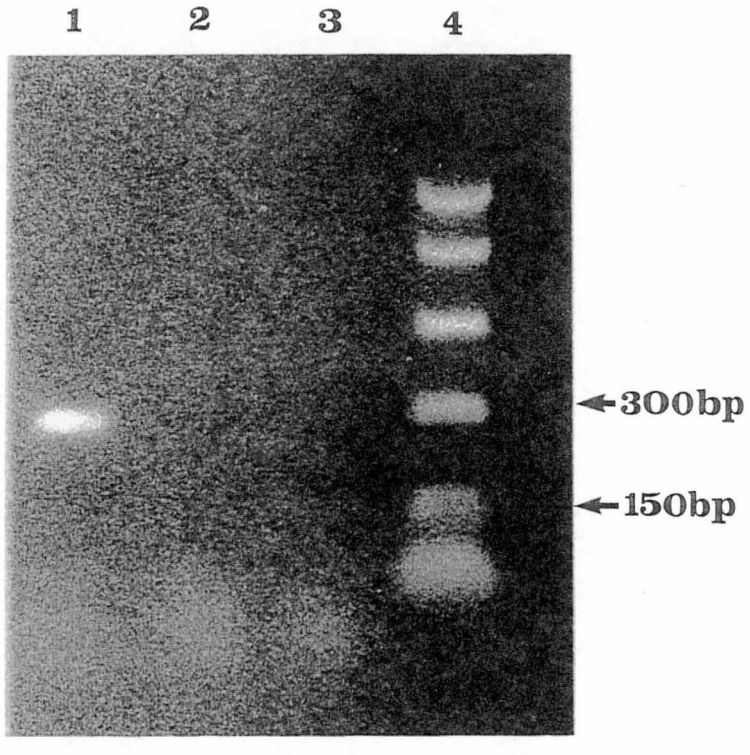
reverse primer

mMT4*Xba*I: 5'-CCtctagaCCCTTGACTCAGGTACTGTG-3'

RT-PCR was performed on total RNA extracted from adult mouse tongue epithelium, using mouse nasal epithelium RNA and no-RNA as negative controls (Fig. 3.11). A PCR product of approximately 290 bp was excised from the gel,

Figure 3.11

PCR was performed at an annealing temperature of 50°C, with primers mMT4*Sac*I and mMT4*Xba*I. PCR products were separated electrophoretically at 80V on 1% agarose/TBE and visualised with ethidium bromide under UV transillumination.



Lane 1: mouse tongue epithelium cDNA + mMT4 primers
Lane 2: mouse olfactory epithelium cDNA + mMT4 primers
Lane 3: no DNA + mMT4 primers
Lane 4: PCR markers

purified and cloned into pUC19 at the *Sac*I and *Xba*I site in a sense orientation. Plasmids were transformed into MC1061, amplified and extracted with alkaline lysis. Four clones were sequenced using the ABI Prism ™ Dye Primer Cycle Sequencing Ready Reaction Kit (Perkin-Elmer Cetus) and found to correspond to the published sequence of mouse MT4 cDNA (Quaife *et al.*, 1994) (results not shown).

3.2.3.2 Screening northern blot of tissues with mMT4 cDNA

To test the conditions under which the mMT4 cDNA will not cross-hybridize with known MT isoforms of *Monodelphis*, plasmids containing each opossum cDNA were doubly restricted with *Xba*I and *Sac*I, the restriction products separated on 1% agarose and blotted onto nylon membrane. Radiolabelled mMT4 cDNA was then used to probe this membrane at varying stringencies. In 50% formamide, mMT4 cDNA hybridized only weakly with all opossum MT cDNA fragments at all temperatures tested below 42°C. Therefore, membrane-bound RNA from various opossum tissues was hybridized with radiolabelled mMT4 in 50% formamide at 42°C, taken to be appropriate conditions under which the mMT4 cDNA might selectively detect an opossum MT4 homolgue.

After exposure to pre-flashed autoradiographic film at -80C° with intensifying screens for six weeks, no signal was apparent in RNA from any tissue (results not shown).

3.2.3.3 Summary

These experiments gave no indication of the occurrence of an *MT4* homologue in the opossum.

3.2.4 Cloning of metallothionein from the Australian echidna, *Tachyglossus aculeatus*

3.2.4.1 Non-exhaustive screen of liver cDNA library gives two distinct MT sequences

A cDNA λgt10 library constructed from hepatic RNA of the Australian echidna, *Tachyglossus aculeatus*, was kindly supplied by Prof. G. Schreiber of the University of Melbourne, Australia.

53,000 recombinant phage were screened using radiolabelled MdMT cDNA at 55°C in 50% formamide. Five positive clones were then sub-cloned into vector pUC19 at the *EcoR*1 site. Clones were sequenced in both directions with the ABI™ Dye Primer Cycle Sequencing Ready Reaction kit (Perkin-Elmer Cetus). Two clones proved to be different full length class I metallothionein cDNAs. Nucleotide sequences are presented in Fig. 3.12.

3.2.4.2 Derived amino acid sequences of echidna MTs

The derived amino acid sequences for two echidna metallothioneins are given in Fig. 3.12, and compared with those of the opossum and the mouse in Fig. 3.8.

Both metallothioniens are comprised of 63 amino acids and contain twenty positionally-conserved cysteines. The single residue insertion at position 3a of the primary sequence (Fig. 3.8) corresponds to the single residue insertion characteristic of eutherian MT3 and MT4, and the documented metallothioneins of birds and amphibia. Like *Monodelphis*, echidna metallothioneins have a single proline insertion at α-domain site 52a, corresponding positionally to the 4-6 residue insertion that characterises eutherian MT3. The carboxyterminal residues of both metallothioneins are glutamine, in common with putative *Monodelphis* isoforms B1 and x5, mammalian MT3s and fish MTs.

There is total conservation of amino acid sequence in the α-domain (sites 32-61, Fig. 3.8) between the two echidna MT isoforms. Within the β-domain, on

Figure 3.12

Aligned nucleotide sequences of cDNAs of two metallothionein isoforms of *Tachyglossus aculeatus.* Putative translation start sites and termination codons are underlined and italicised. Putative polyadenylation signals are given in lower case. Corresponding predicted amino acid sequences are given above the nucleotide sequence in IUPAC-IUB code. Primary sequences are identical except at three positions, given in bold.

```
                              M  D  P  Q  D  C  G  C  A  T  G
MTA CAGNTTCGTGCCCAGCAACGATGGACCCTCAGGACTGCGGCTGTGCCACTGGT
MTB CAGCTTCGTGCCCAGCAGCGATGGACCCTCAGAACTGCGGCTGTGCCACTGGT
                                        N

 G  S  C  A  C  A  T  S  C  K  C  K  D  C  R  C  T  S  C
GGCTCTTGTGCCTGTGCGACATCCTGCAAATGCAAAGACTGCCGGTGCACTTCGTGC
GGCTCTTGTACCTGTGCTGGATCCTGCAAGTGCAAAGACTGCCGGTGCACTTCGTGC
          T        G

 K  K  S  C  C  S  C  C  P  V  G  C  A  K  C  A  Q  G
AAGAAAAGCTGCTGCTCCTGCTGTCCGGTTGGGTGTGCCAAGTGTGCCCAAGGT
AAGAAAAGCTGCTGTTCCTGCTGTCCGGTTGGGTGTGCCAAGTGTGCCCAGGGA


 C  V  C  K  E  P  Q  T  E  S  C  S  C  C  H
TGTGTCTGCAAAGAACCCCAGACCGACAAGTGCAGCTGCTGCCAGTGATGTGGGA
TGCGTCTGCAAAGAGCCCCAGACCGACAAGTGCAGCTGCTGCCAGTGATTGCCGA


      *********
ACCTCCTTGTAAATANTATTGTTTCTACAAATTTGGTTTTCACTGTGCTGAAAGT
GAGGAAAAAAACTTCCTTGTTCTTGTAAATACTGTAATTTGTACAAATCCAGTTT
                         *********       +++++++


        *********
GACCTCCTTGTAAATACTATTGTTTCTACAAATNTGGTTTTCACTGTGCTGAAAGT
CCTCTGTCTGAAAGTTTTTACTTTTTTTTTCTTTTTTCTATGAAATGTGGTGACAT
                                       ~~~~~~~~~~


              ~~~~~~~~
GGTTTTTTTTNTACTTTTCTATAACGTTAaataaa
TTGACTGAAaataaa
```

Sequence motif TTTTTCTAT - ~~~~~~~~
Sequence motif TGTAAATA - ********
Sequence motif TTGTAMA - +++++++

the other hand, the two sequences differ at three residues (=90.3% similarity). Overall similarity between the two echidna proteins is 95.1%.

Between the echidna and opossum metallothineins, there are only 1-2 residues that differ in α-domain. Within the β-domain, differences range between 6-11 sites. The similarity between MT protein sequences of the opossum and the echidna ranges approximately from 80% to 89% (Table 3.3).

Sequence compared	DNA-coding	DNA-α	DNA-β	Protein coding	Protein α	Protein β
Md/Md	87-92%	93-98%	53-87%	87-90%	97-98%	77-87%
Ta/Ta	94%	95%	92%	95%	100%	90%
Md/Ta	72-78%	80-86%	43-60%	80-89%	97-99%	82-90%

Table 3.3 Regions of sequence were compared between the five opossum metallothionein isoforms (Md/Md); between the two echidna MT isoforms (Ta/Ta) and between the opossum MT isoforms and the echidna MT isoforms (Md/Ta). Sequences compared were:
i) coding sequence of the cDNA (DNA-coding)
ii) coding sequence of the α-domain (DNA-α)
iii) coding sequence of the β-domain (DNA-β)
iv) amino acid sequence of the protein (protein coding)
v) amino acid sequence of the α-domain (protein α)
vi) amino acid sequence of the β-domain (protein β)
DNA similarity is calculated from genetic distances derived using the PHYLIP package (as described in Chapter 5), converting dissimilarity into similarity by the function: similarity = (1- dissimilarity) (Swofford *et al.*, 1996), and expressing as a rounded percentage. Amino acid similarity is calculated by simple inspection and rounded to the nearest integer.

3.2.4.3 Classification of derived *Tachyglossus* MT isoforms

3.2.4.3.1 Primary protein structure of predicted echidna MTs

Like the predicted metallothionein molecules of the opossum, the predicted echidna MTs have both the serine residue equivalent to Ser28 (numbered as in Fig. 8) and the glutamine residue equivalent to Gln46 of eutherian MTs 1 and 2. However, unlike the MTs 1 and 2, echidna metallothioniens share a single residue insertion at site 3a of the primary sequence with eutherian MT3s and MT4s.

Further features in common with MT3 include the single proline insertion at site 52a, where the 4-6 residue insert in the C-terminal domain of the eutherian MT3s occurs. This proline is also conserved in all opossum metallothioneins. Both predicted echidna isoforms also share a carboxyterminal glutamine residue with eutherian MT3s, opossum MTs B1 and x5 and fish MTs.

3.2.4.3.2 Apothionein charges

The apothionein molecules of *Tachyglossus* have calculated charges of +2.29 and +3.29. Although, echidna MTB has a calculated charge identical to that of eutherian MT2 isoforms, no corresponding echidna MT sequence has been isolated that compares with eutherian MT1 in its theoretical charge. Consequently, like the opossum metallothioneins, those of the echidna cannot easily be fitted into the classification of eutherian metallothioneins on the basis of the theoretical charge on the protein (see Table 3.2).

3.2.4.3.3 Tissue distribution

Each of the metallothioneins from *T. aculeatus* described here was isolated from a liver cDNA library. They therefore differ in their expression profiles from those observed for eutherian MT3 and MT4.

3.2.4.3.4 Summary

On the basis of tissue distribution, neither predicted *Tachgyglossus* metallothionein can be likened to eutherian MT3 or MT4. Nor can the primary sequence of either echidna metallothionein be reconciled with their classification as mammalian MT1 or MT2. Therefore, like opossum metallothioneins, those of the echidna cannot be easily accommodated within the system of classification as it currently stands for eutherian mammals.

3.3 DISCUSSION

This is the first description of metallothionein cDNA sequences from marsupials or monotremes. In both groups, metallothioneins occur as a multigene

family. The predicted metallothionein proteins of the opossum, *M. domestica* are comprised of 62 amino acids, and those of the echidna, *T. aculeatus* of 63 amino acids. These proteins all contain twenty cysteine residues conserved at sites characteristic of class I metallothioneins, after adjustments have been made for sequence insertions. The correspondence between the isoforms of the eutherian metallothionein gene family, MT1-4, and the predicted MT isoforms of the opossum and echidna is not immediately clear.

3.3.1 Primary structure of opossum MTs

Primary structural differences between the opossum metallothioneins are largely conservative, apart from the dichotomy of terminal residues. While no functional significance has ever been attached to the terminal residues of the various MT isoforms, the notable conservation evident across isoforms and species groups is indicative of strong selection pressure. The (acidic) glutamine and (basic) histidine residues at this position of opossum MTs will affect the overall charge and pKa of the molecule. The calculated charge on the apothionein molecule varies across opossum isoforms, which may affect the capacity for metal-binding, cellular localisation and molecular interaction.

3.3.2 Non-mammalian features of MTs

The amino acid sequences derived from the metallothionein transcripts of *M. domestica* share features with non-mammalian metallothioneins. Carboxyterminal residues of putative opossum MTs are characteristic of either birds (histidine) or fish (glutamine) MTs. The insertion of a single proline at residue 52a (Fig. 3.8) is also typical of the bird metallothioneins (Wei & Andrews, 1988; Fernando & Andrews, 1989; Lin *et al.*, 1990a, 1990b; Lin & Huang, 1990; Shartzer *et al.*, 1993). The derived amino acid sequences of MT transcripts of *T. aculeatus* display carboxyterminal glutamines in common with fish and some opossum MTs, and share the proline insertions at site 52a. Furthermore, the predicted echidna MT proteins have a single glutamine insertion at site 3a, identical to that which characterises the bird

metallothioneins. Precedence for non-mammalian characteristics in marsupial and monotreme proteins are reported in marsupial neurotensin (Shaw *et al.*, 1991), the protamine P1 proteins of marsupials (Retief *et al.*, 1995) and monotremes (Retief *et al.*, 1993), monotreme milk lysozymes (Teahan *et al.*, 1991), marsupial transthyretin (Duan *et al.*, 1995), transferrin (Lim *et al.*, 1987; Lim *et al.*, 1988) and the transferrin receptor (Lim *et al.*, 1987), insulin-like growth factor I (Yandell *et al.*, 1998), hypothalamo-neurohypophyseal hormones (Hurpet *et al.*, 1982) and gonadotrophin-releasing hormone (King *et al.*, 1989).

3.3.3 Structure of the gene family

The predicted metallothioneins of the opossum are an homogeneous group of sequences: amino acid differences between the opossum MTs are few and usually conservative, and no insertions or deletions occur relative to one another. All transcripts are expressed in both the brain and the liver. While no transcripts corresponding more closely to eutherian MT3 or MT4 isoforms have been recovered, the absence of these sequences from the opossum genome has not been conclusively established. Neither has the extent of the gene family been fully explored. Chromosome walking has been necessarily applied to the clarification of the *MT* locus in mouse genome (Quaife *et al.*, 1994), and may extend our knowledge of opossum MTs, should the genes be tandemly aligned as they are for other mammals. Any conjecture which may be made about the structure of the *Monodelphis MT* gene family must accommodate these limitations. It is tempting to equate the predicted *Monodelphis* metallothioneins with the eutherian MT1 and MT2 molecules, on several grounds: duplication of the genes of these isoforms is common in eutherian mammals, giving rise to clusters of very similar sequences; tissue distribution is broad; and these sequences were historically more readily accessible. However, it is perhaps premature to draw such a conclusion. The taxonomic group most closely related to the mammals, the Aves, has a very simple metallothionein gene family with a single highly-conserved metallothionein protein (Lin *et al.*, 1990b; Lin & Huang 1990; Shartzer *et al.*, 1993), or in the case of the pigeon, two (Lin *et al.*, 1990a).

The ancestor common to both mammals and birds, therefore, can be presumed also to have had a simple metallothionein family. The elaboration of the family in the eutherians may have no exact analogy in the marsupials. That there has been multiple duplication of the metallothionein genes in both mammalian subclasses (and possibly also in the monotremes), and not within other vertebrate groups, may give some insight into the nature and aetiology of this amplification. The history of the metallothionein multigene family may, in turn, clarify the role of the metallothioneins in organismal fitness.

3.3.4 Marsupial MT3 and MT4

Efforts were made to isolate from the opossum the cDNA of a cognate protein with characteristics specific to the MT3 and MT4 isoforms of eutherian mammals. These characteristics include recognised conserved sequences for each isoform and tissue-specific patterns of expression. No such cDNA was isolated. The strong sequence conservation among the vertebrate *MT* genes creates difficulties in their isolation (Searle *et al.*, 1984; Huang *et al.*, 1998), as is often the case for the members of mulitgene families (Ardley *et al.*, 1998; Kel *et al.*, 1998). Furthermore, the tissue-specificity of an isoform need not necessarily be conserved across taxonomic groups. Therefore, the occurrence (or not) of an MT isoform corresponding to mammalian MT3 or MT4 in the opossum has not been established. The mammalian MT3 and MT4 isoforms were themselves identified much later in the course of metallothionein research than the MT1 and MT2 isoforms (Uchida *et al.*, 1991; Palmiter *et al.*, 1992; Tsuji *et al.*, 1992; Quaife *et al.*, 1994), when the extent of the various mulitigene families had been presumed fully documented (eg. West *et al.* 1990).

3.3.5 Expression

Both brain and liver cDNA libraries were constructed from RNA taken from animals which had not been treated to induce metallothionein expression. It is unlikly that the time interval between removal of the opossum from its cage and sacrifice was sufficient to allow stress-induced transcription. Therefore, it

can be inferred that all five of the metallothionein cDNAs described here represent genes that are constitutively transcribed in the opossum. It remains to be demonstrated that these opossum genes are also expressed at the protein level.

Six metallothionein proteins have been isolated from normal adult human livers (Hunziker & Kagi, 1985), implying their simultaneous expression. At least four metallothionein transcripts are known to be co-expressed in the uninduced sheep liver, though only MT2 mRNA was detectable in cultured sheep fibroblasts without induction (Peterson & Mercer, 1988). Extensive expansion of the metallothionein gene locus is, therefore, often reflected in a sizeable complement of constitutively-expressed hepatic MTs. This work demonstrates that a proportion of genes at the duplicated marsupial metallothionein locus is also transcribed in both brain and liver, without induction.

3.3.6 Sequence conservation

3.3.6.1 Domains

Sequence conservation is high in the α-domains of the predicted metallothioneins of the opossum and echidna (Table 3.3). The β-domain is far less constrained at both the gene and protein levels. This implies either the direct action of selection pressure in response to some structure-function limitation in the α-domain or alternatively, the selective action of gene conversion upon the α-domain to maintain sequence homogeneity. It may be inferred that conservation of the protein is more critical in the carboxyterminal α-domain than in the β-domain. Greater conservation of the primary sequence of the α-domain than the β-domain is also true for members of the metallothionein gene families of the mouse (Searle *et al.*, 1984) and sheep (Peterson *et al.*, 1988), as well as for the vertebrate gene family as a whole (see Kagi & Schäffer, 1988). It has been suggested (Peterson *et al.*, 1988) that conservation of the α-domain of metallothionein, which binds zinc in preference to copper, reflects the physiological importance of MT in metabolic zinc homeostasis.

3.3.6.2 5' untranslated regions

Fewer than 60 nucleotides of 5' untranslated region (UTR) upstream from the translation initiation codon were cloned for the opossum metallothioneins. There is marked conservation between four of the five opossum metallothionein cDNAs in the proximal twelve nucleotides of the 5' UTR (92%), which compares to 42% between mouse MT1 and MT2, and 75% between mouse and human MT2s (Searle *et al.*, 1984) in the same region. The fifth opossum cDNA, L3, shows only 50% homology with the other opossum MTs over this sequence. Sequence conservation in the 5' UTR of the opossum MT cDNAs does not extend beyond the proximal twelve nucleotides. Only the proximal twenty bases of the 5' untranslated region of the echidna metallothionein cDNA molecules have been cloned and these show exceptional sequence conservation of 90% (possibly 95%, given uncertainty at one nucleotide). It is unfortunate that the full extent of this conservation within the 5' untranslated region has not been established.

3.3.6.3 sequence motifs of the 3' untranslated regions

The putative polyadenylation signal AATAAA (Proudfoot & Brownlee, 1976) occurs at varying distances from the translation termination codon in the opossum and echidna MT cDNA molecules. The 3' UTRs of the two echidna metallothioneins show negligible homology (conserved motifs excepted). Some homology is demonstrable for the proximal 12 bases of the opossum cDNAs, beyond which meaningful alignment is impossible.

The sequence TTTTCTAY (Y=pyrimidine) has been noted in the 3' UTR of various eutherian metallothoinein 1 and 2 genes (Peterson *et al.*, 1984; Peterson *et al.*, 1988; Kobayashi *et al.*, 1997), twenty nucleotides upstream from the polyadenylation signal. These motifs do not occur in the *MT3* genes of mouse (Genbank M93310) or human (Genbank M93311) or the documented mammalian *MT4* genes (Quaife *et al.*, 1994). Similar sequences, with or without a single base change, occur in four of the opossum metallothionein transcripts at

variable distances from the polyadenylation signal (Fig. 3.13; Table 3.4). The L3 sequence of the opossum has a closely related motif, but with a single base insertion TTTTTC**C**TAT, occurring 32 bases before the putative polyadenylation signal.

isoform	sequence	bp 5' from AATAAA
B1	(T)TTTT**A**TAC	52
	(T)TTTTC**C**AC	20
	TTTTCTA**A**	32
B3	(T)TTTTCTAT	56
	(T)TTTTCTAT	67
L2	(T)TTTTC**A**AT	17
	(T)TTTTCT**T**T	87
L3	(T)TTTTC**C**TAT	32
x5	(T)TTTT**A**TAC	62

Table 3.4 - Variants of the conserved sequence motif TTTTCTAY occurring in the 3' UTRs of the five metallothionein isoforms of *M. domestica*. Single base differences are given in boldface. (T) represents sequence usually conserved in the opossum, but not always in eutherian MT cDNAs.

The motifs TTTTCTAT and (T)TTTTCTAT occur at 15 and 33 base pairs from the polyadenylation signal of echidna metallothioneins A and B respectively (Fig. 3.12). These are identical to the eutherian motif TTTTCTAY, and closely resemble those noted above in the opossum metallothioneins.

A second conserved sequence that occurs in the 3' UTR of metallothioneins 1 and 2 genes of the hamster (Griffith *et al.*, 1983), mouse MT1 (Durnam *et al.*, 1991), human MT2 (Karin & Richards, 1982) and dog MT (Kobayashi *et al.*, 1997) is TGTAAATA, 24-26 base pairs downstream from the termination codon. This motif occurs in each opossum MT sequence 20-21 nucleotides from the end of the coding region, with a single base change at position 5 in three of the isoforms (TTGTA**G**ATA) (Fig. 3.13). (This substitution differs from the base change that occurs in the human version of the motif [Griffith *et al.*, 1983] but occurs at the same location as the base change in canine MT cDNA [Kobayashi *et al.*, 1997]). The fully conserved sequence TGTAAATA occurs in both echidna metallothionein clones, 15 and 31

```
B3                  CCTCCTTCTGCTGTGAGACCTGCATCCCGACATCTATCTGCCAGCCATGGACCCCAACTGTGACTGCGTGAGCGGAAGTTCTTGCACC
L2                                GAGAGGTTAACGTCTCCCTGCCAGCCATGGACCCCGACTGTGACTGCGTGAGCGGTGGCTCTTGCACC
L3                      CTTGCTCGACCCGCAGAGAGAACTCCAGACTTCCTAGTTATGGACCCCAACTGTAACTGCGCCAACGGTGGCTCCTGCACC
B1                CCATCCTTCTCCTGTGAGCTCTGCGTGCCTGACGTGTACCTGCCAGCCATGGACCCCAAATGCAACTGCGAGAGTGGAGGTTCTTGCACC
X5  CAACCTTCCAGCCCATCCTTTTCCTCTGAGACTTGAGCACCTGCATCTACCGGCCAGCCATGGACCCCAATTGTGACTGTGGGAATGGTGCCTCTTGTACC

B3   TGTGCAGGCTCCTGCAAGTGTAAATCCTGCCGATGTACCTCCTGCAAGAAAAGCTGCTGCTCCTGCTGCCCAGCGGGATGTGCCAAGTGTGCCCAGGGCTGTG
L2   TGTGCAAACTCCTGCAAATGTAAATCTTGTCAGTGTACCTCCTGCAAGAAAAGCTGCTGCTCCTGTTGCCCAGTGGGATGTGCCAAGTGTGCTCAGGGCTGTG
L3   TGCGGAGACTCTTGCAAATGCAAATCATGCTCCTGTACCTCCTGTAAGAAAAGCTGCTGCTCCTGCTGCCCAGTGGGATGTGCCAAGTGTGCGCAGGGCTGTG
B1   TGTGCAGGCTCCTGCAAATGTAAATCCTGCCGCTGCACCTCCTGCAAAAAAAGCTGCTGCTCCTGCTGCCCAGTGGGATGTGCCAAGTGTGCCCAGGGCTGTG
X5   TGTGCAGGCTCCTGCAAATGTGCATCCTGTCACTGTACTTCCTGCAAGAAAAGCTGCTGCTCCTGTTGCCCAGTGGGATGTGCCAAGTGTGCCCAGGGCTGTG

B3   TCTGCAAAGCCCCCCAGACTGAGAGTTGCAGCTGCTGCCACTGATGCTCCTCTCTCCCATGACTGTAAATAGAAGAATTTGTACAGACTTGACTTTTCATACAC
L2   TCTGCAAAGCCCCCCAGACTGAGAGTTGCAGCTGTTGCCACTGATGCTCCCATTTTTCTTTGCCTGTAAATAGAAGTTGTACAGACCTGAATTATCCTATACCA
L3   TCTGCAAAGCCCCCCAGACTGAGAGTTGCAGCTGTTGCCACTGATGTTCCCACCTCCTCATTCCTGTAGATAGAAGATGCCATCTGTTTTTGTGCACCATGAAT
B1   TCTGCAAAGCCCCCCAGACTGAGAGTTGCAGCTGCTGCCAGTGATGTTCCTGTCTTCTTGTGCCTGTAGATAGAGGAATTTGTACAGACCTGATTTTTTTATAC
X5   TCTGCAAAGCCCCCCAGACTGAGAGTTGTAGCTGCTGTCAGTGATGGACCTGCCCCCCTGTACTTGTAAATAGAGGAATTTGTTCAGATGTGGCTTTTTATACA

B3   TTTGGTCATATTTTTCTATTTTTTTTCTATAAATGTGTGATTCAATCCACAGGCATTTATTAACCACTTAGTATGCCaataaa
L2   AAGATATGTATTCTACTGTTTTTCAATGAAGCATGaataaa
L3   GTTTTTCTACTGTTTTTCCTATGAAATGTATAAATGAAaataaa
B1   ACTGAGTTGTGTTTTTCTAATCTTTTTTCCACTATGAAATGAAaataaa
X5   CTGTAGATGTACTTTCTGCTTTGTTTCCATGAAATTTGTGAATGAAaataaa
```

Figure 3.13 Aligned sequences for five *Monodelphis* metallothionein cDNAs.

Sequence motifs corresponding to TTTTTCTAY - TTTTTCTAY

Sequence motifs corresponding to TGTARATA - TGTARATA

Sequence motifs corresponding to TTGTACA - TTGTACA

nucleotides downstream from the coding region in *MTA* and *MTB*, respectively. It is repeated in the echidna MTA cDNA, 71 bases from the coding region. This motif does not occur in the 3' untranslated regions of human MT3 gene (Genbank M93311) or mouse *MT4* (Quaife *et al.*, 1994). However, it is present in the mouse *MT3* gene (Genbank M93310), 27 nucleotides beyond the termination codon.

A third sequence motif is identified here in three of the five opossum sequences of the 3' UTR, TGTACA, at a site 33-36 nucleotides from the termination stop codon in transcripts B1, B3 and L2. This motif, with a single base mismatch, also occurs at the same position in transcript x5 (Fig. 3.13). The 3' UTR of the L3 cDNA molecule contains the altered motif TGT**G**CA at a slightly removed position (Fig. 3.13; Table 3.5). TGTACA is further conserved in the echidna metallothionein *MTB* gene, 46 nucleotides from the coding region (Fig. 3.14). Examination of Genbank entries indicates that TGTACA occurs in the 3' UTR of *MT2* in mouse, human, sheep, rat, hamster (with a single base change but positionally correct) and the human *MT2* processed pseudogene. The sequence does not occur in the 3' untranslated sequences of *MT1* in mouse, hamster, sheep, rabbit, pig, monkey, bovine or human (with two exceptions) (Table 3.5).

Sequence	TGTACA site (from termination codon)	Genbank accession no.
human MT1a	-	K01383
human MT1b	-	M13485
human MT1e	-	M10942
human MT1f	40 bp	M10943
human MT1g	-	J03910
human MT1h	-	X64834
human MT1k	-	Stennard *et al.*, 1994
human MT1r	-	X972612
human MT1x	72 bp	X65607
human MT2	61 bp	J00271
human MTψ2	59 bp	M13074
human MT3	-	M93311
human MT4	84 bp	U07807
mouse MT1	-	V00835

mouse MT2	60 bp	K02236
mouse MT3	-	M93310
mouse MT4	-	U07808
rat MT1	-	M11794
Rat MT2	58 bp	M11794
rat MT3*	-	Y08235
rabbit MT1	-	X07790
pig MT1	-	M29515
pig MT3	-	U95969
monkey MT1	-	K00484, V01533
monkey MT2*	-	K00485, V01532
bovine MT1	-	M76977
dog MT1	35 bp	D84397
hamster MT1	-	D10551
hamster MT2§	TGTAGA - 37 bp	J03848
sheep MT1a	-	X04626
sheep MT1b	-	X07973
sheep MT1c	-	X07974
sheep MT2	40 bp	X07975
opossum MTB1	37 bp	this thesis
opossum MTB3	36 bp	" "
opossum MTL2	34 bp	" "
opossum MTL3	TGTGCA - 48bp	" "
opossum MTx5	TGTTCA - 37 bp	" "
echidna MTA	-	" "
echidna MTB	46 bp	" "

Table 3.5 - The occurrence within the mammals of the 3' UTR sequence motif TGTACA.
* sequence of the 3' UTR is incomplete
§ chinese hamster MT2 sequences D10552, J00062 do not contain a 3' UTR TGTACA motif

No corresponding sequence is found in the 3' UTR of mouse, rat or human *MT3*. No such motif occurs in mouse *MT4*, though it does so in human *MT4*. The signature occurs in no 3' UTR sequence of bird *MT*.

These data suggest that the 3' UTR sequence motif TGTACA is specific to *MT2* sequences. The only exceptions found in the Genbank database are human *MT4*, dog *MT1*, human *MT1x* and human *MT1f* which contain the sequence; and monkey *MT2* which does not. Interestingly, though human MT1f does not carry an acidic amino acid at sites 10 or 11 that would justify its

classification as an MT2, the apothionein molecule has a charge of +3.29 consistent with most eutherian MT2 molecules (Table 3.2).

If the 3' UTR sequence motif TGTACA is considered specific to *MT2* transcripts, three opossum MT cDNAs may be grouped on this basis with the eutherian *MT2*s. The x5 and L3 cDNAs each contain a degenerate motif, and that of the L3 molecule is offset from the site conserved in the other four. Further analysis of the possible role of this motif in transcript regulation is necessary before these two variants are deemed authentic motif homologues, although the positional conservation of the x5 motif lends it added credibility. The echidna B metallothionein sequence also contains the conserved motif, and can therefore be grouped with the *MT2*s on this basis. It is noteworthy that in all mammalian metallothionein multigene families so far documented, including the opossum and echidna, at least one member of the liver-derived mRNA molecules contains this sequence signature and another does not.

Of the five reported opossum MT transcripts, three (B1, B3, L2) share all three conserved motifs in the 3' UTR, and strong conservation in the twelve proximal bases of the 5' UTR. The x5 transcript shares two motifs and strong conservation of the 5' UTR. The L3 transcript, on the other hand, shares only one non-degenerate motif in the 3' UTR and departs markedly from conservation of the 5' UTR. It may be that the regulation of the L3 transcript differs in some way from that of the other opossum metallothionein mRNAs. The expression of individual opossum MT transcripts is discussed in Chapter 4.

Short conserved sequences in untranslated regions are construed to play a regulatory role in transcript management, at the level of translation (Standart & Jackson, 1994; Lai & Posakony, 1997), tissue specificity (King *et al.*, 1994; Wolford & Signs, 1995), cellular compartmentalisation of the transcript (Kislauskis *et al.*, 1993, 1994; Havin *et al.*, 1998) and localisation of the protein (Kislauskis *et al.*, 1994), post-transcriptional repression (Schuelke *et al.*, 1998) and mRNA stability (Kastelic *et al.*, 1996; Holcik & Liebhaber, 1997; Leviten *et al.*, 1997; Dandekar *et al.*, 1998). The first two sequence signatures described above occur virtually exclusively across a range of metallothionein genes

(Griffith *et al.*, 1983), suggesting a metallothionein-specific function. The third motif, TGTACA, appears to occur specifically in the 3' untranslated region of eutherian *MT2* and some marsupial and monotreme sequences, implying a role in the regulation of this transcript. Preferential localisation of mouse MT1 mRNA over MT2 mRNA to the perinuclear region of cultured hepatoma cells, and a concomitant association with the cytoskeleton, has been reported (Mahon *et al.*, 1995). This phenomenon is proposed to be a precondition for post-translational transport of the cognate protein into the nucleus. The control of differential compartmentalisation of transcripts has been imputed to regulatory sequences in the 3' untranslated regions of the MT1 transcript (Mahon *et al.*, 1997). It can be inferred, therefore, that conserved elements exist in the 3' UTRs of these molecules that differentiate the disposition of *MT* gene products in the cell. Although it has been suggested that relatively long 3' UTR sequences (50-200 nucleotides) are required for compartmentation of mammalian transcripts (St. Johnston, 1995), secondary structure of the RNA is thought to contribute to the mechanism (Hesketh, 1996). The *MT2*-specific sequence noted above, though short, is palindromic and therefore likely to support secondary structure within the region. Furthermore, when the motif-containing transcripts are examined, the spacing between the second (TGTAAATA) and third (TGTACA) motifs becomes significant. In one echidna and 3 opossum sequences, sheep MT2 mRNA, and that of hamster MT2 and human MT1f, the TGTA of the second motif forms a stem with the TACA of the third motif which supports a 14-base loop. In human, mouse or rat MT2 mRNA, this loop is 35, 31, 32 bases respectively. TGTACA has also been documented as an androgen-response element, usually occuring in the 5' UTR or intron sequences (Tsai *et al.*, 1988; Devos *et al.*, 1997).

3.3.7 Summary

In summary, the metallothionein transcripts of the opossum and the echidna are similar to the eutherian MTs 1 and 2 in several ways: tissue distribution, putative regulatory sequences, some features of the cognate protein.

However, other characteristics liken them to eutherian MT3s: site-specific insertions, carboxyterminal residues and a range of charges on the cognate proteins. Further clarification is necessary before a confident classification of opossum and echidna metallothioneins can be made.

CHAPTER 4: EXPRESSION of METALLOTHIONEIN mRNA in *MONODELPHIS DOMESTICA*

4.1 Introduction

Metallothionein is expressed or can be induced in virtually all tissues of the eutherian mammal (Chen & Ganther, 1975). Tissue specificity is characteristic of the MT3 and MT4 isoforms (Tsuji *et al.*, 1992; Quaife *et al.*, 1994), but the MT1 and MT2 isoforms occur in a broad range of cell and tissue types. The tissue distribution of the five metallothionein transcripts expressed in *Monodelphis* was investigated to allow a comparison with the expression profiles of the eutherian MT genes. This was intended to extend the general comparison of eutherian and marsupial metallothioneins, and to advance the classification of the marsupial sequences.

The ontogenic expression of eutherian MTs, especially in the liver and brain, is well documented. Embryonic and fetal levels of hepatic metallothionein are very high, and peak either during gestation for species with well developed neonates (Klein *et al.*, 1991) or perinatally in species with precocial young (Bakka & Webb, 1981). In contrast, low levels of metallothionein occur in the fetal brain, and concentrations begin to rise at birth to maximum levels in the adult (Masters *et al.*, 1994b; Holloway, 1996; Holloway *et al.*, 1997a). Whether these developmental profiles correlate with organismal maturation or with environmental factors that precipitate physiological changes at birth has not been established.

The developmental profile of the marsupial is very different from that of the eutherian mammal. Gestation is short and the young are born at a level of maturity that conforms in many ways to the eutherian embryo (Johnson, 1977). Birth, therefore, largely precedes the maturation process. Consequently, the marsupial offers a propitious model in which to dissect the consequences of birth upon metallothionein expression from the effects of physiological maturation. The postnatal "embryonic" marsupial is also divorced to a large extent from maternal influences that may modify metallothionein expression.

4.1.1 Opossum development

The South American opossum has a gestational period of 14 days (Fadem *et al.*, 1982). The placenta is non-invasive and attaches only superficially in the final days of gestation (Krause & Cutts, 1984, 1985, 1986). Active morphogenesis is restricted to the last six days before birth (Krause & Cutts, 1986). At birth, the young opossum has been compared to the fetal rat at 13 days post-conception (Saunders *et al.*, 1989). A developmental mosaicism exists in the opossum neonate, however, insofar as some features are necessarily highly developed to allow its independent existence, while others are relatively immature. So the comparison is an approximation. Neonates are very small: 10mm in length and 100mg in weight (Kraus & Fadem, 1987) (Fig. 1.6c). At birth, they crawl to a teat on the maternal abdomen where they remain attached for 14-21 days. There is precocious development of breathing and digestive systems (Saunders *et al.*, 1989). The forelimbs are capable of rhythmic, alternating movements at birth (Cassidy *et al.*, 1994) which may be initiated in the spinal chord (Pfleiger *et al.*, 1996), while the hindlimbs are vestigial buds incapable of independent movement. The neonatal opossum therefore displays a gradient of maturation along the axis of its central nervous system (Møllgard *et al.*, 1994).

The marsupial newborn is ectothermic (Petajan & Morrison, 1962) and the metabolic rate is correspondingly low. Oxygen consumption in the major organs, including the liver and brain, increases steadily from birth until thermoregulation is achieved (Hulbert, 1988). The acquisition of homothermy in marsupials is more accurately equated with eutherian birth than is emergence from the pouch, as the latter varies across species (Tyndale-Biscoe & Janssens, 1988). Endothermy often coincides with weaning in the marsupial (Tyndale-Biscoe & Janssens, 1988) and the opossum is weaned 50-60 days after birth (Saunders *et al.*, 1989; Clark & Smith, 1993; Robinson & Dooley, 1995). The period of most rapid growth in the opossum is between postnatal days 30 -100 (Saunders *et al.*, 1989). *Monodelphis* continues to grow up to 200-250 days whereupon the growth rate slows considerably, though the opossum increases in size and weight throughout its life (Cothran *et al.*, 1985).

Neocortical development of eutherian and marsupial mammals follows a similar pattern and a similar time course (Saunders *et al.*, 1989). The former, however, occurs *in utero* and the latter postnatally. The brain of the opossum neonate has been described as "embryonic" (Johnson, 1977), and is comparable in its maturity to that of the rat at 14 days post-conception or the six week human fetus (Saunders *et al.*, 1989). At birth, the opossum forebrain has an unstructured, two-layered "embryonic" form (Saunders *et al.*, 1989). The cerebellum is absent (Nicholls *et al.*, 1990) and the olfactory bulb extremely immature (Cassidy *et al.*, 1994). A rudimentary choroid plexus is present in the lateral ventricles (Reynolds & Saunders, 1988). Very little brain growth occurs in the first two days after birth, and most neurogenesis occurs in the second and third weeks of life (Clark & Smith, 1993). Maturation of the neocortex is apparent by 45 days after birth and is characteristic of the adult brain at postnatal day 60 (Saunders *et al.*, 1989). The marsupial brain continues to grow throughout life, though this is less likely to involve an increase in neurons as an enhancement of other brain components such as astrocytes, dendritic processes and extracellular matrix (Nelson, 1988)

To better compare the predicted opossum metallothioneins with other mammalian MTs, the expression of *Monodelphis* MT transcripts in various tissues was examined. The opossum neonate differs markedly from the eutherian neonate. To evaluate whether these differences are reflected in the perinatal expression of metallothionein, MT mRNA levels were examined in the brain and liver of the newborn opossum.

4.2 RESULTS

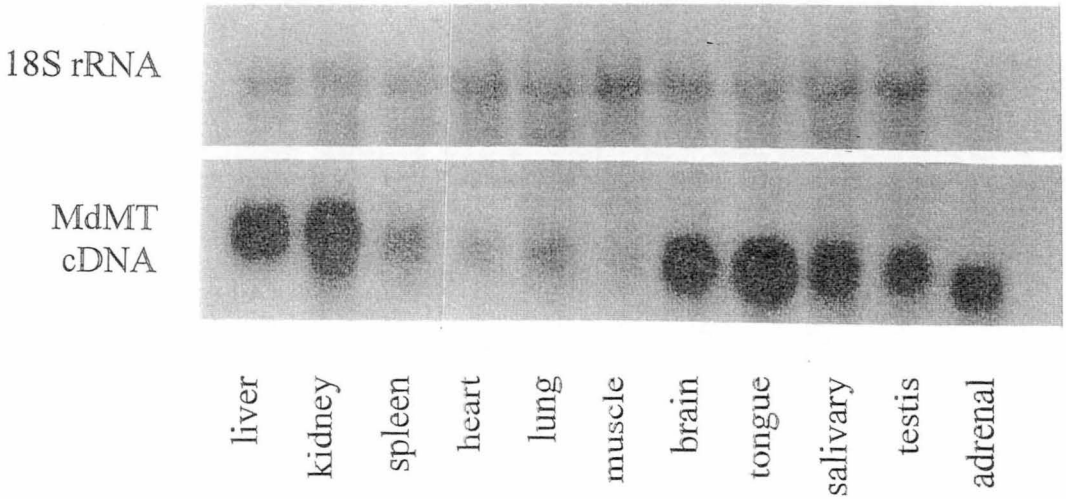
4.2.1 Detection of MT mRNA in tissues by northern analysis

4.2.1.1 Tissue distribution of metallothionein in the adult opossums

Total RNA was isolated from the tissues of adult opossums (n=3) and pooled. RNA was separated electrophoretically through a 1% denaturing agarose gel and blotted onto nylon membrane. Membranes were probed with

Figure 4.1

Total RNA was extracted from adult opossums (n=3) and pooled. RNA was separated electrophoretically on a 1% denaturing agarose gel and blotted onto nylon membrane. The membrane was probed with radiolabelled MdMT cDNA. To establish the relative amount of RNA in each lane, the membrane was also probed with an 18S rRNA-directed oligonucleotide.



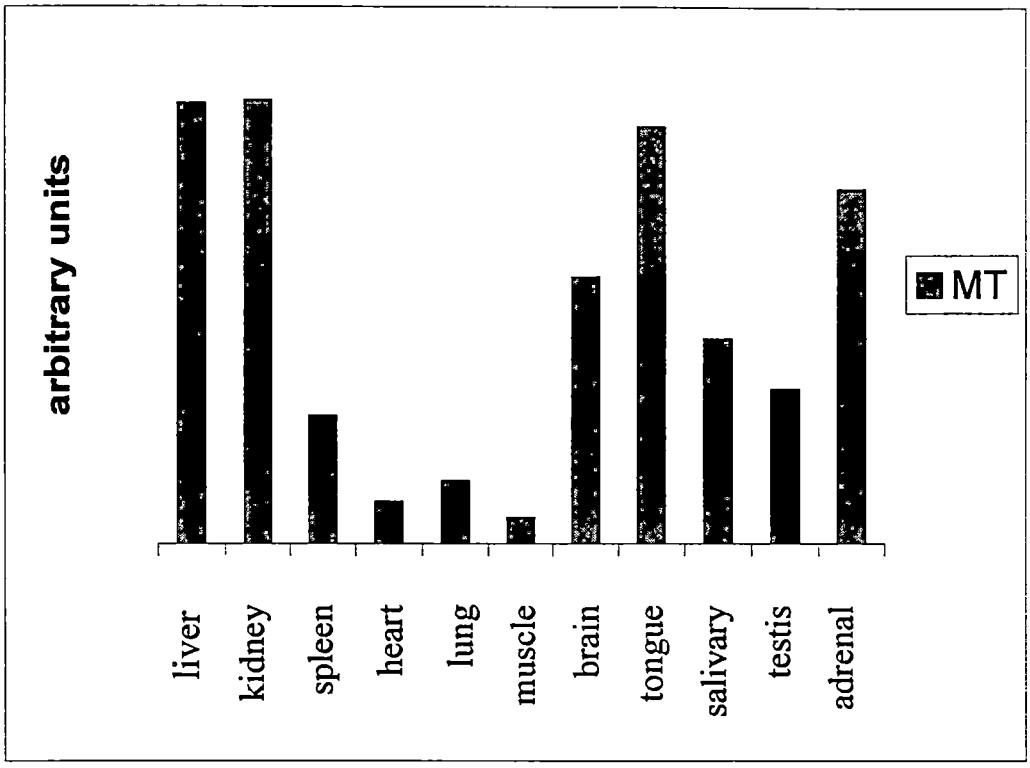


Fig. 4.2

Relative levels of MT mRNA in tissues of the opossum, *M. domestica*. Data from Fig. 4.1 were corrected for any differences in RNA per lane and results graphed using Excel.

radiolabelled MdMT cDNA probe (described in section 2.7.1; Fig. 3.3) to ascertain levels of metallothionein mRNA in each tissue (Fig. 4.1). A radiolabelled oligonucleotide probe to 18S rRNA (section 2.7.2) was used to establish the relative amounts of RNA in each lane. Autoradiographic bands were quantified with densitometry and adjustments made for differences in the amount of total RNA per lane. Histograms are presented in Fig. 4.2.

Metallothionein transcripts may exist in the opossum that do not bind the MdMT cDNA probe at the stringency used in this experiment. Interpretation of these results must be made with this in mind. The MT cDNA molecules described in Chapter 3 were each isolated using the MdMT cDNA probe, and are therefore known to hybridize at the stringency used here.

Basal levels of metallothionein mRNA in the opossum are highest in the liver and the kidney (Fig. 4.2). Interestingly, the level of MT mRNA in the tongue is almost as high. The adrenal gland also displays a very high level of metallothionein transcript. Brain, salivary gland and testis express moderate levels of MT mRNA. Expression in the spleen, heart, lung and muscle is considerably lower.

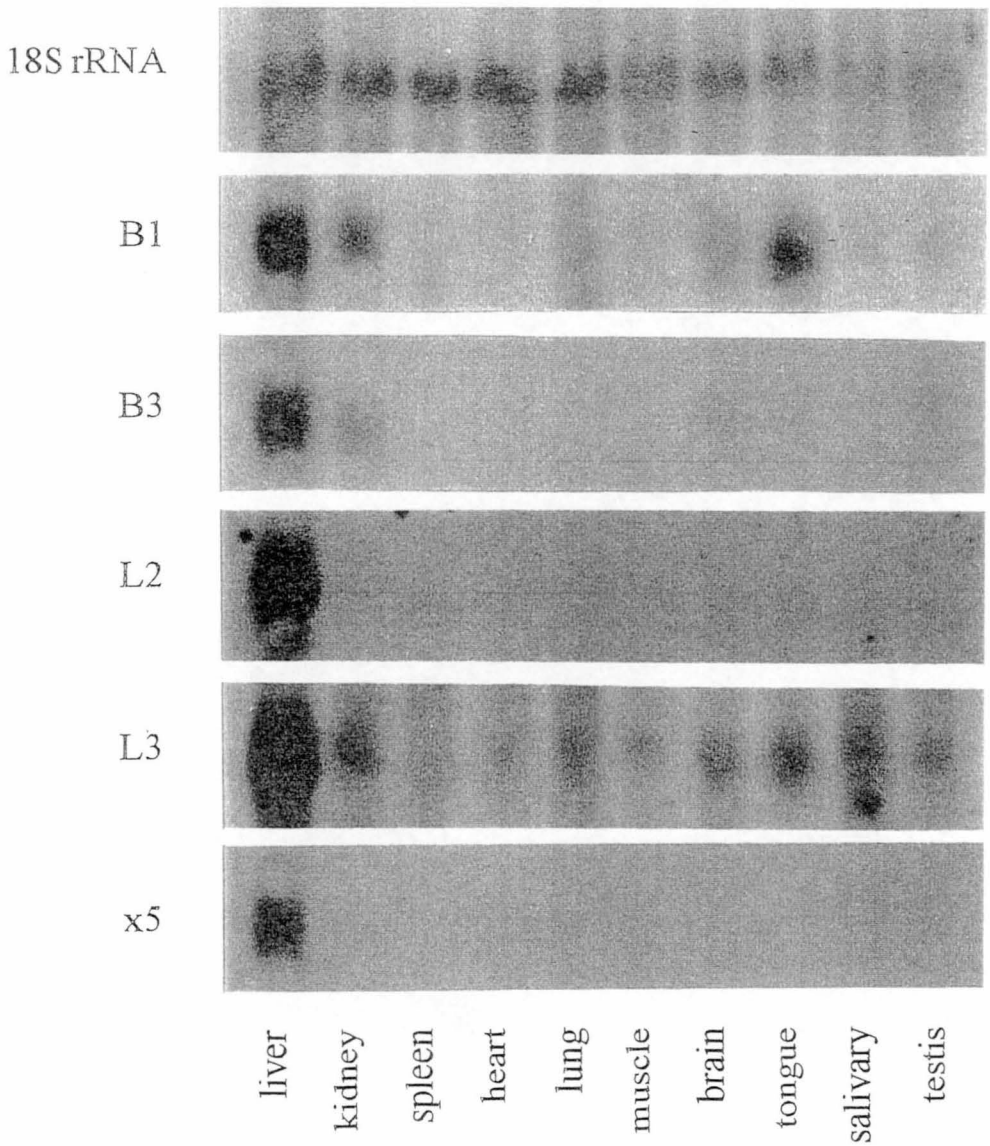
4.2.1.2 Tissue distribution of different MT transcripts in adult opossum

Total RNA was isolated from the tissues of adult opossums (n=3) and pooled. RNA was separated electrophoretically through a 1% denaturing agarose gel and blotted onto nylon membrane. Membranes were probed with radiolabelled oligonucleotide probes specific for the transcripts identified in Chapter 3 (the design of these probes is described in section 3.2.1.3; the probe sequences in section 2.7.2; and the probe sites in Fig. 3.4 and Fig. 3.7b) to establish tissue-specific expression for each transcript (Fig. 4.3). Autoradiographic bands were quantified with densitometry and adjustments made for differences in the amount of total RNA per lane using a probe for 18S rRNA.

Tissue-specificity is clearly a factor in the transcription of these opossum metallothionein genes (Fig. 4.4). Each of the metallothionein sequences described for the opossum is transcribed most intensely in the liver. With the

Figure 4.3

Total RNA was extracted from adult opossums (n=3) and pooled. RNA was separated electrophoretically on a 1% denaturing agarose gel and blotted onto nylon membrane. The membrane was probed with radiolabelled oligonucleotides specific to the five opossum MT cDNAs. To establish the relative amount of RNA in each lane, the membrane was also probed with an 18S rRNA-directed oligonucleotide.



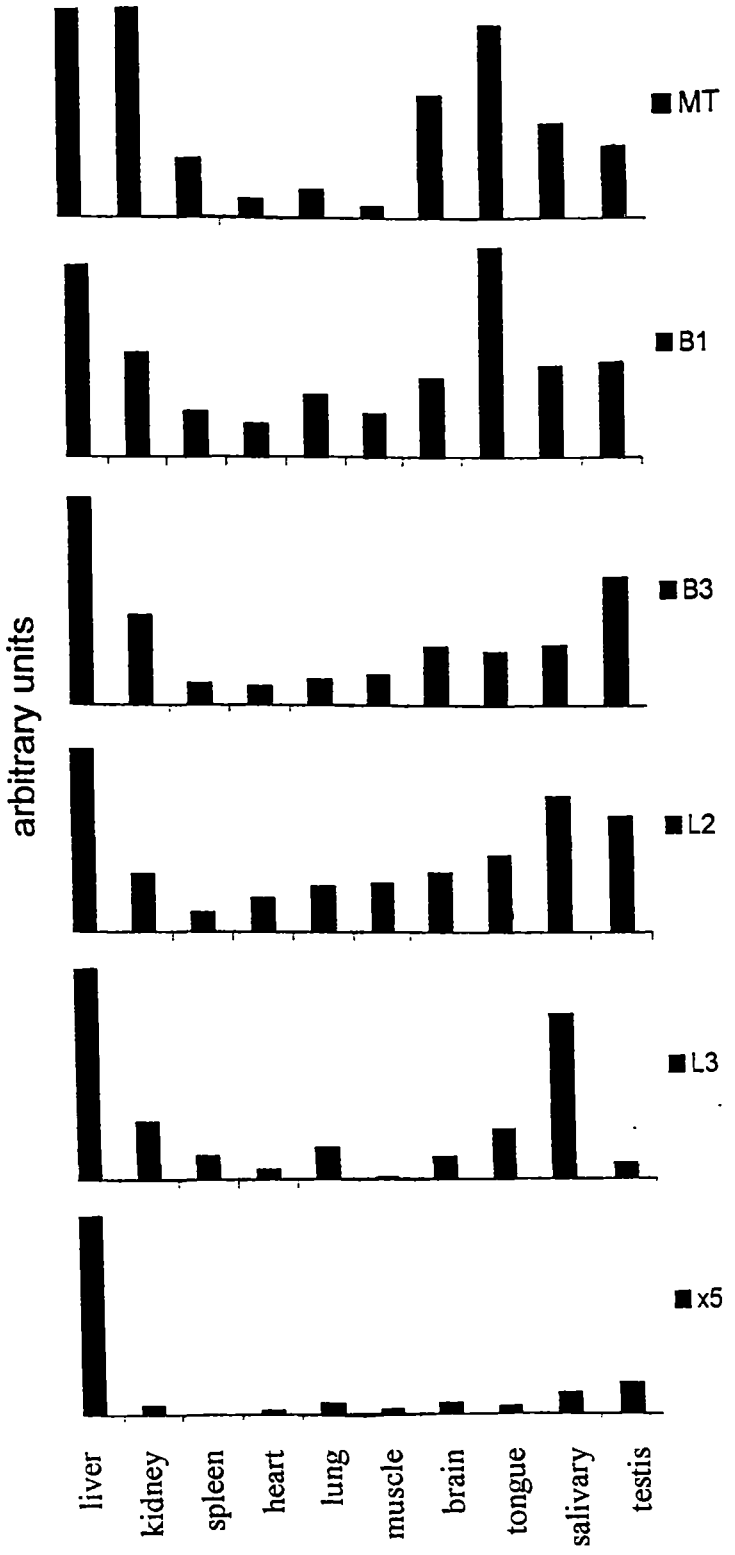


Fig. 4.4

Relative levels of MT mRNA in the various tissues of the opossum, *M. domestica*. Topmost histogram: combined levels of transcript detected with the MdMT cDNA probe (from Fig. 4.1). Remaining histograms represent levels of each individual transcript, derived from the data of Fig. 4.3, after correction for differences in total RNA per lane. Results were graphed using Excel.

exception of x5, each transcript is also expressed highly in another tissue type. B1, B3 and L2 mRNA have expression profiles very similar to the profile detected with the generalised MdMT cDNA probe, known to detect all five transcripts. The tissue distribution of L3 and x5 mRNA is much more restricted. Because it is not possible to assess the comparative degree to which each transcript is expressed, the transcript complement within each tissue cannot be defined from these data.

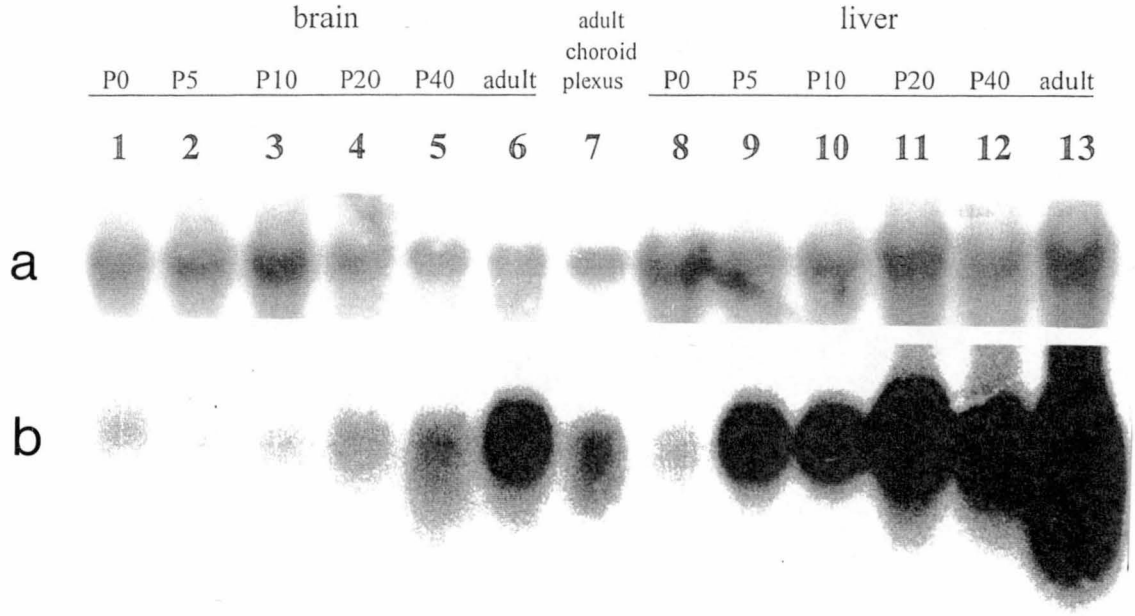
4.2.1.3 Pattern of MT mRNA expression in development

Total RNA was extracted from opossum neonatal livers and brains at birth (postnatal day 0 = P0, n=10), at postnatal day 5 (P5, n=5), at P10 (n=2), at P20 (n=2), at P40 (n=1) and from adult animals (n=1). At P0, the neonate is too small to easily dissect out the brain and liver, so for this time point, whole head and whole body (minus the head) tissues were used respectively in their stead. Total RNA was also extracted from the adult choroid plexus and included in this analysis to compare specific levels in the choroid plexus with those in the complete brain. The RNA was separated electrophoretically on a denaturing agarose gel, blotted onto nylon membrane and probed with radiolabelled MdMT cDNA, which corresponds to the internal 40 amino acids of *Monodelphis* cDNA MT L3 (Chapter 3). Autoradiographic bands were quantified with densitometry, and adjustments made for any differences in the amount of total RNA per lane, using a probe to 18S rRNA. Autoradiographs are presented in Fig. 4.5 and adjusted values in Fig. 4.6.

4.2.1.3.1 Liver

There is a steady increase in hepatic metallothionein expression in the opossum from birth to adult. No peak is evident, comparable to the peak of MT expression that occurs in the eutherian fetus or neonate. The basal level of MT transcript is lower in the opossum head than in the body at birth, whereas MT levels in the brain are higher than in the adult liver. All eutherians examined to date have a fetal/neonatal peak in hepatic MT expression, when MT mRNA concentrations exceed adult levels (eg. Andrews *et al.*, 1984; Lehman-

Figure 4.5.
MT mRNA in opossum brain and liver through developmental stages



RNA was extracted from total brain and liver tissue at various ages of the neonatal and adult opossum, and also from the adult choroid plexus. RNA was separated electrophoretically on a 1.2% denaturing agarose gel, blotted onto nylon membrane and probed with
a. the oligonucleotide probe against 18S rRNA
b. the MdMT cDNA probe (described in Fig. 3.3 and section 2.7.1).

Lane 1: RNA extracted from head at P0
Lane 2: RNA extracted from brain at P5
Lane 3: RNA extracted from brain at P10
Lane 4: RNA extracted from brain at P20
Lane 5: RNA extracted from brain at P40
Lane 6: RNA extracted from adult brain
Lane 7: RNA extracted from adult choroid plexus
Lane 8: RNA extracted from body (minus head) at P0
Lane 9: RNA extracted from liver at P5
Lane 10: RNA extracted from liver at P10
Lane 11: RNA extracted from liver at P20
Lane 12: RNA extracted from liver at P40
Lane 13: RNA extracted from adult liver

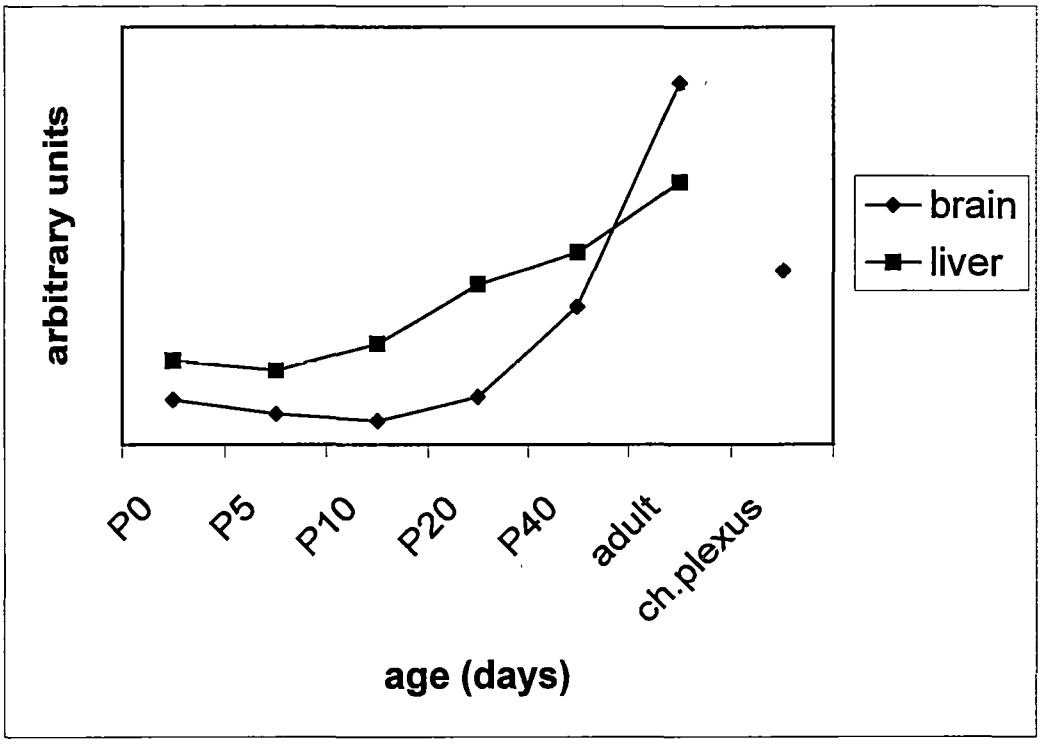


Fig. 4.6

Levels of MT mRNA in brain and liver at various postnatal ages in the opossum, *M. domestica*. MT mRNA level in the adult choroid plexus is also shown. Data are derived from Fig. 4.5 and graphed using Excel.

McKeeman *et al.*, 1988a). Maximum expression of hepatic MT mRNA in the opossum, however, occurs in the adult.

4.2.1.3.2 Brain

Brain metallothionein mRNA in the opossum rises from low levels at birth to high basal levels in the adult. Very little change occurs before P10 and the greatest increase occurs after P25. This is comparable to findings in the eutherian brain. Significant levels of MT transcript are present in the choroid plexus, though at levels below that of total brain.

4.2.2 Detection of MT mRNA in tissue by *in situ* hybridization

In situ hybridization was performed, as described in section 2.13, on several tissues of the adult opossum using the ^{32}P-labelled cDNA probe MdMT (this probe is described in section 2.7.1 and Fig. 3.3). Brain, tongue and salivary gland were included among the tissues studied to investigate further the high level of transcript expression in these tissues demonstrated by northern analysis. Mouse uncoupling protein I (*UCP1*) cDNA was used as negative control. The expression of uncoupling protein is restricted to brown adipose tissues in eutherian mammals (Cannon *et al.*, 1982). Neither brown adipose tissue (Gemmell & Cepon, 1993; Nicol *et al.*, 1997) nor uncoupling protein (Loudon *et al.*, 1985; Ye *et al.*, 1995: Hope *et al.*, 1997;) has been identified in non-macropod marsupials.

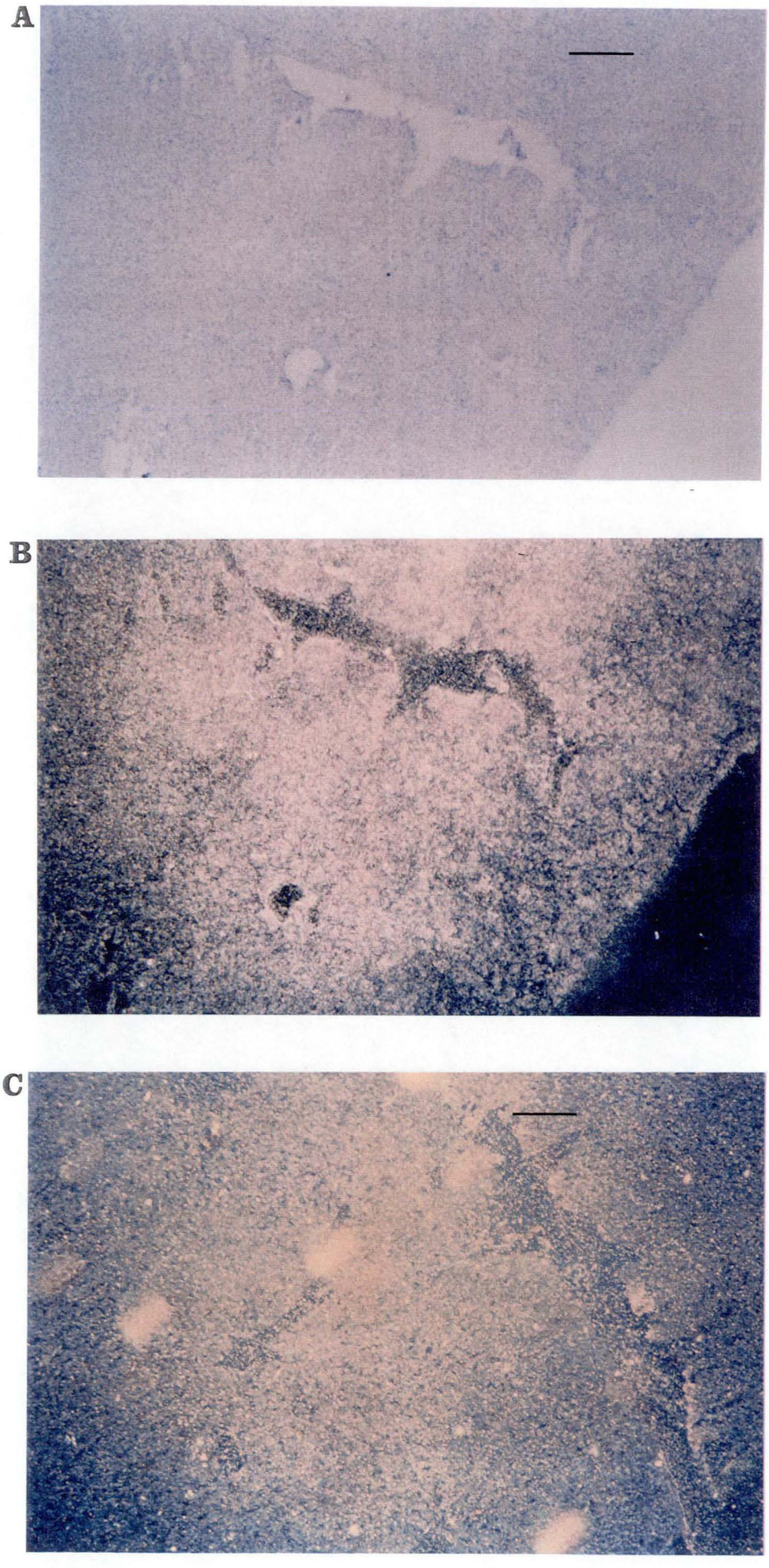
4.2.2.1 MT mRNA expression in liver

The distribution of metallothionein mRNA in the liver is very generalised, implying its presence in hepatocytes (Fig. 4.7). There appears little or no localised enhancement of transcript expression. The relative low intensity of the background signal in the negative control becomes more obvious when the extended time of exposure is considered (MdMT = 35.3s; neg. control UCP = 6.67 min).

Fig. 4.7 LIVER

Paraffin sections (0.7 μm) of opossum liver were hybridised with ^{32}P-labelled cDNA probes. Sections are stained with haematoxylin.

A. Section of opossum liver was hybridized with ^{32}P-labelled MdMT cDNA. Bar = 400μm.
B. Corresponding dark field shows strong homogeneous signal, signifying a high level of MT mRNA expression throughout the liver, presumably in hepatocytes. Exposure time = 35.3s.
C. Dark field exposure of a control section of opossum liver, hybridized with mouse UCP1 cDNA. An exposure time of 6.67 min was necessary to achieve this signal intensity.
Bar = 400μm


A
B
C

4.2.2.2 MT mRNA expression in kidney

MT mRNA expression in the opossum kidney is extensive (Fig. 4.8) and confirms the very high levels of constitutively expressed MT mRNA evident in the northern analysis of Fig. 4.1. Within the renal cortex, a higher level of MT mRNA expression can be localised to the tubules (larger arrows) associated with the glomeruli (small arrows) in Figs. 4.9A, B. It is not possible to precisely identify these ducts but their positions relative to the glomeruli suggest they are distal and proximal convoluted tubules, as described in Diagram 4.1

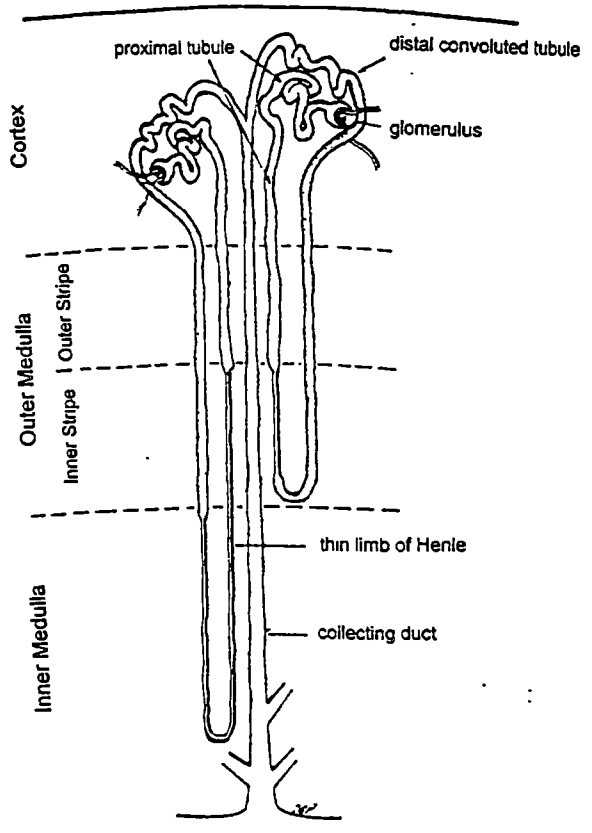


Diagram 4.1: Structural organisation of the renal nephron (filtering unit), modified from The Kidney, vol. I - B.M. Brenner & F. C. Rector (eds) (1965).

The glomeruli themselves do not express MT mRNA (Fig. 4.9A, B - small arrows). Within the medulla (Fig. 4.9C, D), MT mRNA is localised to the thin

Fig. 4.8 KIDNEY

Autoradiographs of longitudinal paraffin sections (0.7 μm) of opossum kidney hybridized with ^{32}P-labelled cDNA probes. Sections are stained with haematoxylin.

A. An overview of the kidney, showing the renal cortex (R), lumen (L) and the renal medulla (M). Section was hybridised with ^{32}P-labelled MdMT cDNA. Bar = 400μm.
B. Corresponding dark field shows intense signal throughout the cortex and medulla, relative to background levels in the renal lumen. Localised areas of more intense signal occur throughout both the cortex and the medulla. These are investigated at high magnification in Fig. 4.9. Exposure = 1.3 min.
C. Dark field exposure of a control section, hybridized with mouse UCP1 cDNA. An exposure time of 7.8 min was necessary to achieve this signal intensity.
Bar = 400μm

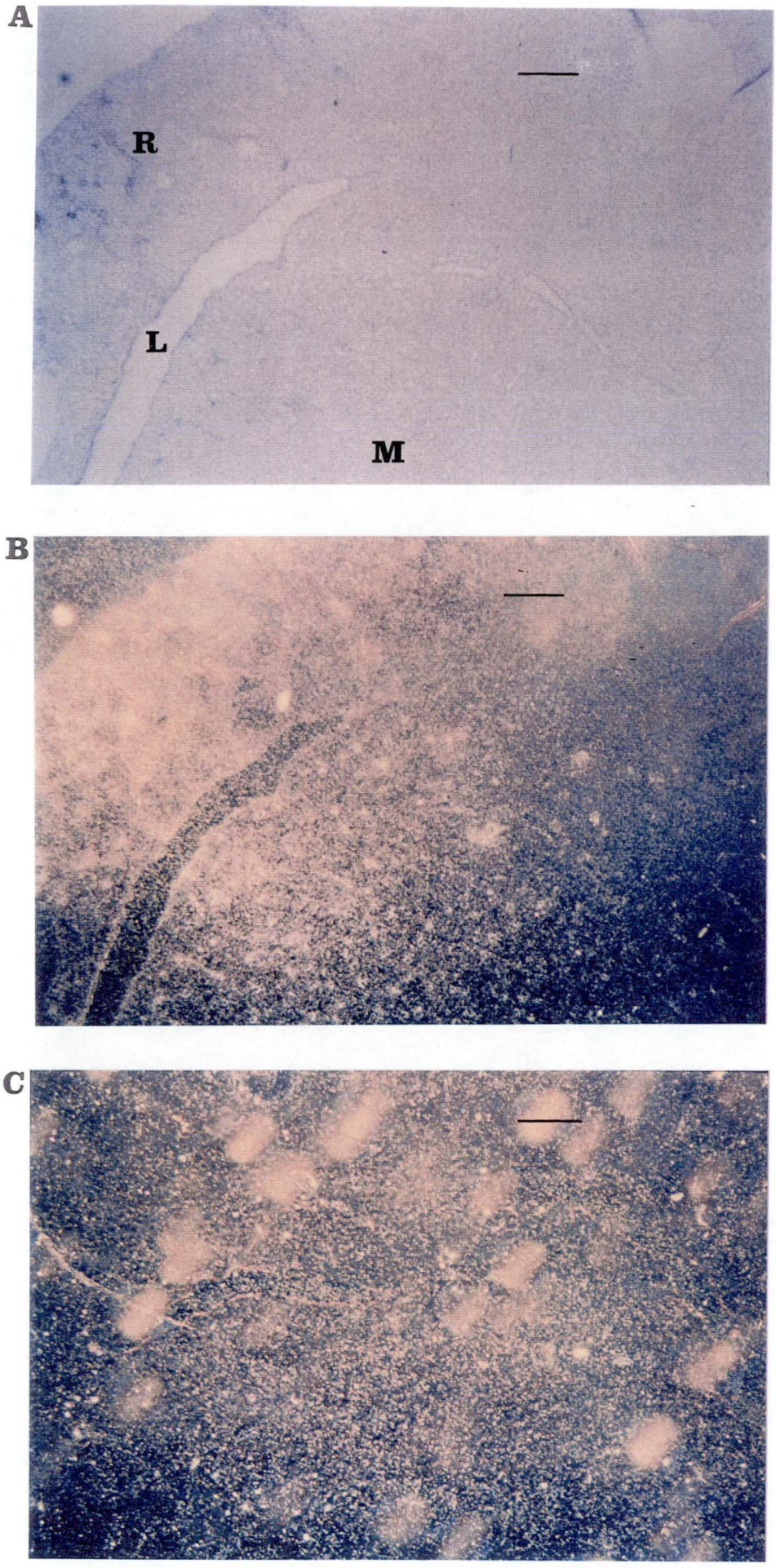
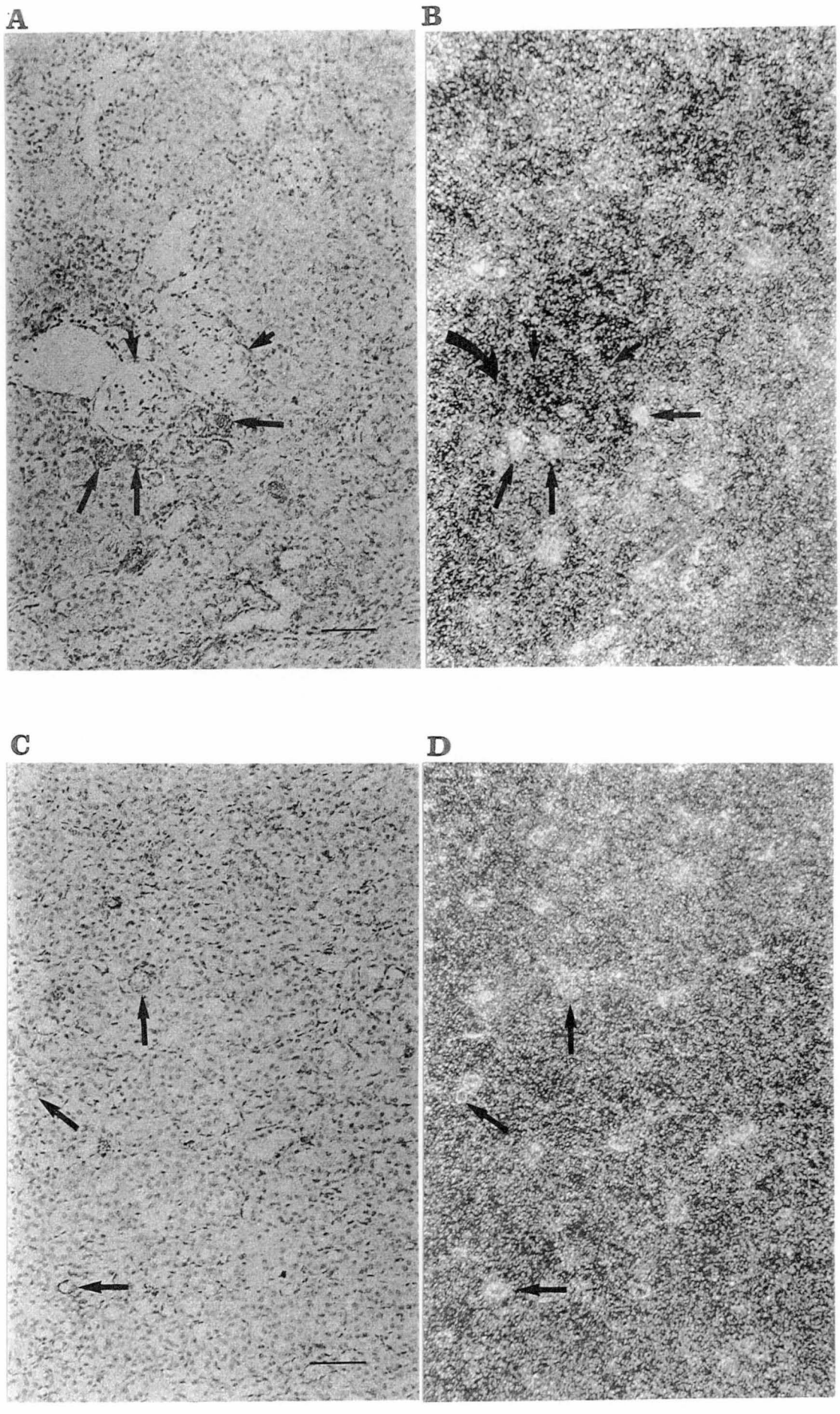

A
R
L
M
B
C

Fig. 4.9 KIDNEY

Autoradiographs of longitudinal paraffin sections (0.7 μm) of opossum kidney hybridized with ^{32}P-labelled MdMT cDNA. Sections are stained with haematoxylin.

A. Section through the renal cortex shows glomeruli (small arrows) and surrounding ducts (larger arrows), presumed by their position to be distal and proximal convoluted tubules.
   Bar = 160μm
B. Corresponding dark field exposure shows no signal in the glomeruli (small arrows) above the background visible in the adjoining empty space (curved arrow). Intense signal corresponds to distal convoluted tubules (arrows).
C. Section through the renal medulla shows narrow ducts identified as the thin limbs of Henle (arrows).
   Bar = 160μm
D. Corresponding dark field exposure shows intense signal in the thin limbs of Henle (arrows) indicating MT mRNA expression in the ductal epithelium. Strong generalised signal in the medulla is attributible to MT transcription throughout this region which is composed principally of aggregated tubules.


A
B
C
D

limbs of Henle, described in Diagram 4.1. MT mRNA is evidently in the tubular epithelium of these ducts.

4.2.2.3 MT mRNA expression in brain

Coronal sections through the forebrain of the opossum were prepared as described and probed with radiolabelled MdMT cDNA. Sections shown in Figs. 4.10-12 are equivalent to plate 11 in The Brain of the Opossum (*Didelphis marsupialis*) by Oswaldo-Cruz & Rocha-Miranda (1968). The gross cerebral anatomy and histology of *M. domestica* is very similar to that of the North American opossum *Didelphis* (Schwanzel-Fukuda *et al.*, 1988). Figs 4.10A,B and 4.11A,B show very intense expression of MT mRNA in the ependymal layer of the lateral and third ventricles. This is localised to the single layer of ependymal epithelial cells (Fig. 4.10C, D). Diffuse signal occurs throughout both the hippocampus and the hypothalamus (Fig. 4.10, 4.11). Within the periventricular grey matter about the third ventricle (Fig. 4.11), isolated spots of very intense signal signify high levels of MT transcript within the epithelium of blood vessels and in the region immediately surrounding them (Fig. 4.11C, D). This surrounding signal appears to derive from MT transcript in the astrocytes that circumscribe these capillaries, forming part of the blood-brain barrier.

The choroid plexus displays a level of MT transcript expression considerably lower than that of the ependyma (Figs. 4.10A, B; 4.12A, B). These findings confirm the northern analysis of Fig 4.5, which indicates significant levels of MT mRNA in the adult choroid plexus.

MT mRNA in the hippocampus appears as a strong generalised signal when compared to the background levels of signal in the lumen of the lateral ventricle (Fig. 4.12A, B). The cells of the granular layer of the dentate gyrus (small arrows) appear to express higher levels of metallothionein transcript than do the cells of the surrounding white matter of the molecular layers (larger arrows).

Control sections hybridised with radiolabelled mouse UCP1 cDNA (Fig. 4.12C, D) show no signal above the level of background seen in the lumen of the lateral ventricle, nor localisation of intensified signal to any particular cell type, such as the ependymal layer of the ventricle. It was necessary to use very fast

Fig. 4.10 BRAIN

Coronal sections of opossum midbrain were hybridised with ^{32}P-labelled MdMT cDNA. Paraffin sections (0.7μm) were stained with haematoxylin.

A. Lateral ventricle, showing a segment of choroid plexus (arrow), the granular layer of the dentate gyrus (small arrows) and the surface of the cortex (curved arrow). The orientation of the hippocampus in the marsupial brain is orthogonal to that in the rat (Harman, 1997).
   Bar = 500μm
B. Corresponding dark field shows intense signal in the ependymal cells lining the lateral ventricle. There is low but distinct signal in the choroid plexus (arrow). Expression of MT mRNA is slightly higher in the granular layer of the dentate gyrus than in the surrounding hippocampus (large arrow).
C. At higher magnification, the distinct single layer of ependymal cell layer lining the lateral ventricle (Fig. 4.10A) can be seen (arrow).
   Bar = 125μm
D. Corresponding dark field showing strong MT mRNA expression localised to the single layer of cells of the ependyma. A lower but significant level of MT mRNA is expressed in the periventricular region (pv).

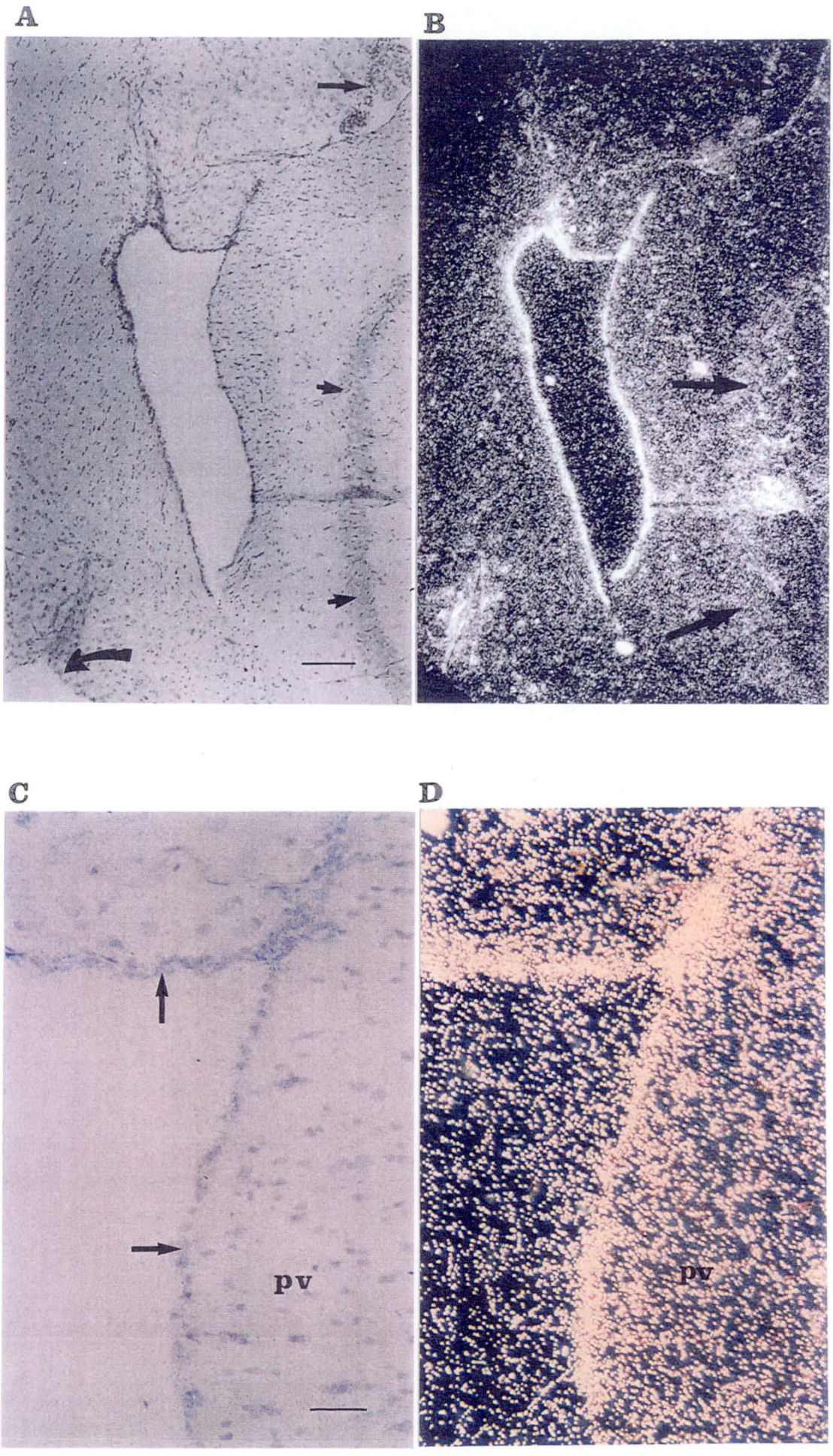

A
B
C
pv
D
pv

Fig. 4.11 BRAIN

Coronal sections of opossum midbrain were hybridised with ^{32}P-labelled MdMT cDNA. Paraffin sections (0.7μm) were stained with haematoxylin.

A. Third ventricle (arrow) within the hypothalamic region (ht), showing the single cell layer of ependymal cells (small arrow). Region described within the rectangle is magnified in frames C and D.
   Bar = 500μm
B. Corresponding dark field shows intense expression of MT mRNA in the ependymal cells of the third ventricle. The level of signal in the hypothalamic region is significantly higher than that of the ventricular lumen, suggesting MT mRNA expression throughout the hypothalamus. Localised intense signal occurs in the periventricular grey matter and an area of this region (delineated in rectangle) is examined under high power in Fig. 4.11C, D.
C. The hypothalamic region surrounding the third ventricle is shown in Fig. 4.11A to express high levels of very localised MT mRNA. The region marked by the rectangle of Fig. 4.11A. is shown here under higher magnification. Intense signal is visible as silver grains (black) and is shown to correspond to capillaries (small arrows) and the area immediately about them (arrows).
   Bar = 50μm
D. Corresponding dark field showing intense localised expression of MT mRNA to capillaries, with a surrounding region of intense signal.

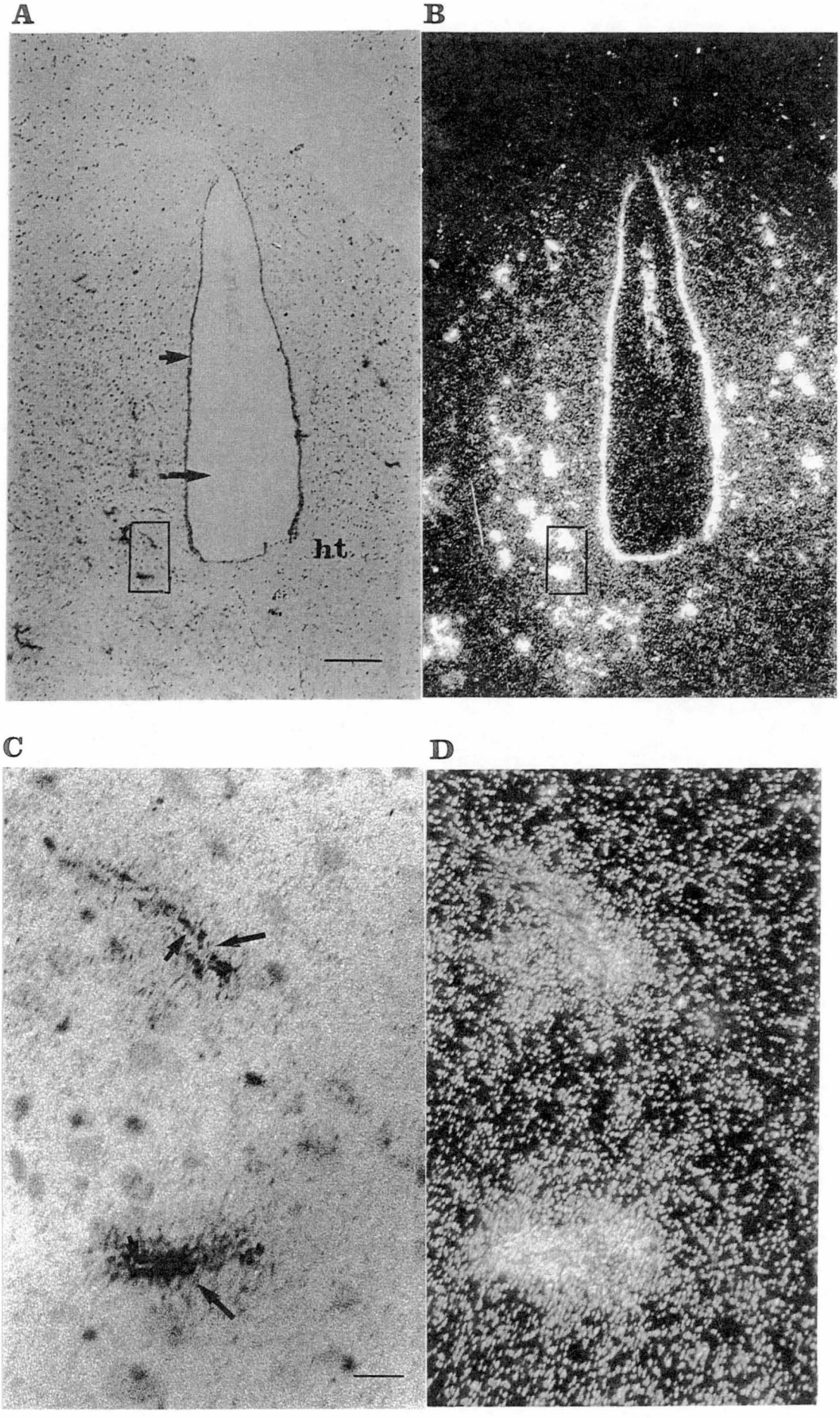
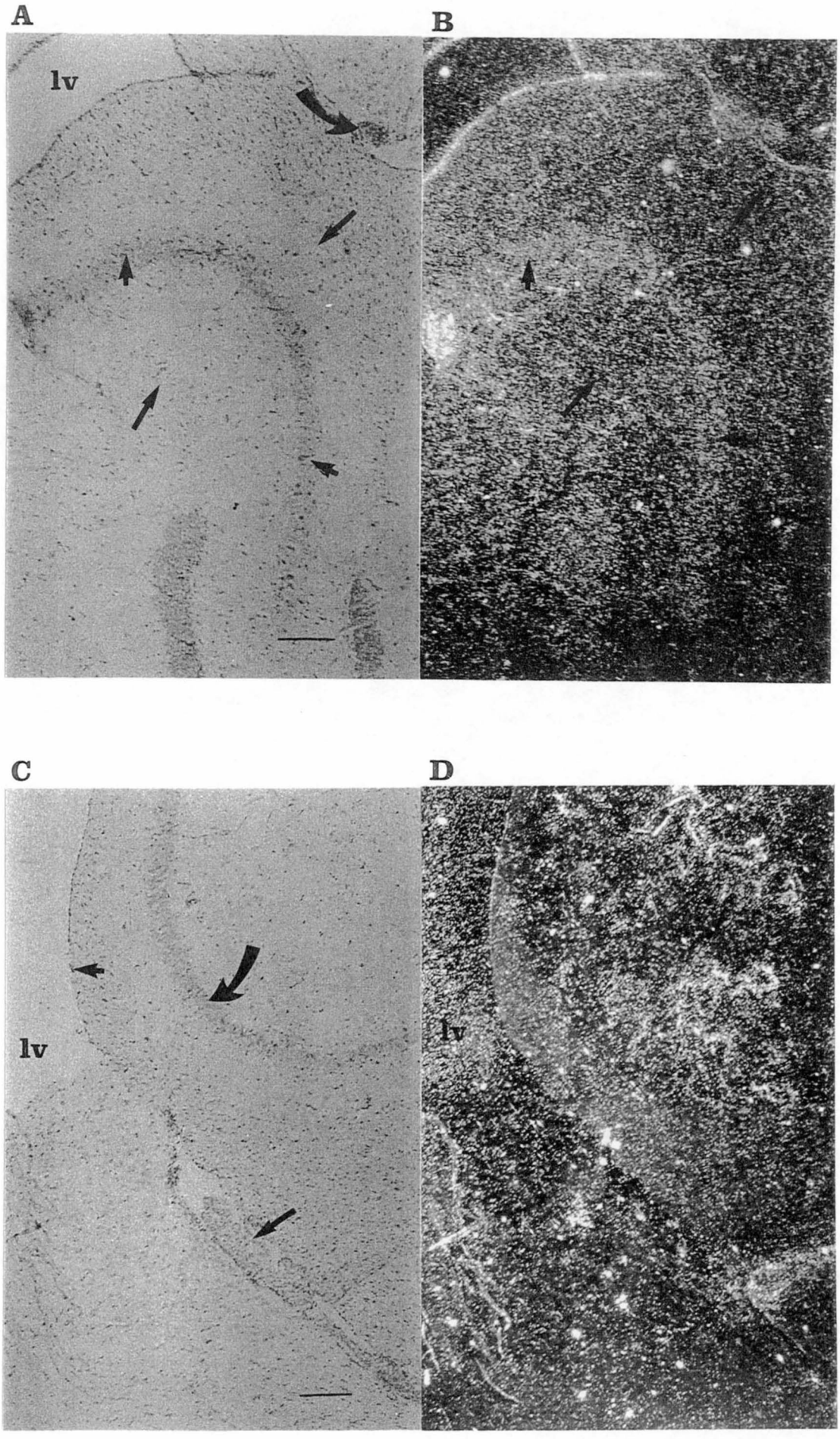

A
B
ht
C
D

Fig. 4.12 BRAIN

Coronal sections of opossum midbrain were hybridised with ^{32}P-labelled cDNA probe. Paraffin sections (0.7μm) were stained with haematoxylin.

A. Coronal section of the forebrain shows the granular (small arrows) and molecular (arrows) layers of the dentate gyrus of the hippocampus in relation to the lateral ventricle (lv) and choroid plexus (curved arrow). Section was hybridised with MdMT cDNA.
   Bar = 500μm
B. Corresponding dark field exposure shows signal throughout the hippocampus is well above the background level displayed in the ventricular lumen, though below that of the ependymal cells lining the lateral ventricle. The granular layer of the dentate gyrus (small arrow) is more strongly positive for MT mRNA than is the molecular layer (arrows).
C. Control section hybridised with mouse UCP1 cDNA, showing ependyma (small arrow) of the lateral ventricle (lv), choroid plexus (arrow), and the granular layer of the dentate gyrus (curved arrows).
   Bar = 500μm
D. Corresponding dark field exposure shows no signal delimiting the lateral ventricle, nor enhanced signal marking the granular layer of the dentate gyrus. Background signal within the ventricular space (lv) is equivalent to the signal over tissue. Fast film (ISO400) was used to achieve this level of signal in frames C and D.


A
lv
B
C
lv
D
lv

film (ISO400) to achieve this level of signal in control sections, whereas sections probed with MdMT cDNA (Fig. 14.12A, B) were photographed with ISO25 film.

4.2.2.4 MT mRNA expression in salivary gland

The epithelium lining the major ducts of the opossum submandibular salivary gland express high levels of metallothionein mRNA (Fig 4.13). Both excretory and striated ducts show intense signal. No signal is apparent in the secretory units (the acini) of the gland above the background level apparent in the lumen of ducts.

4.2.2.5 MT mRNA expression in tongue

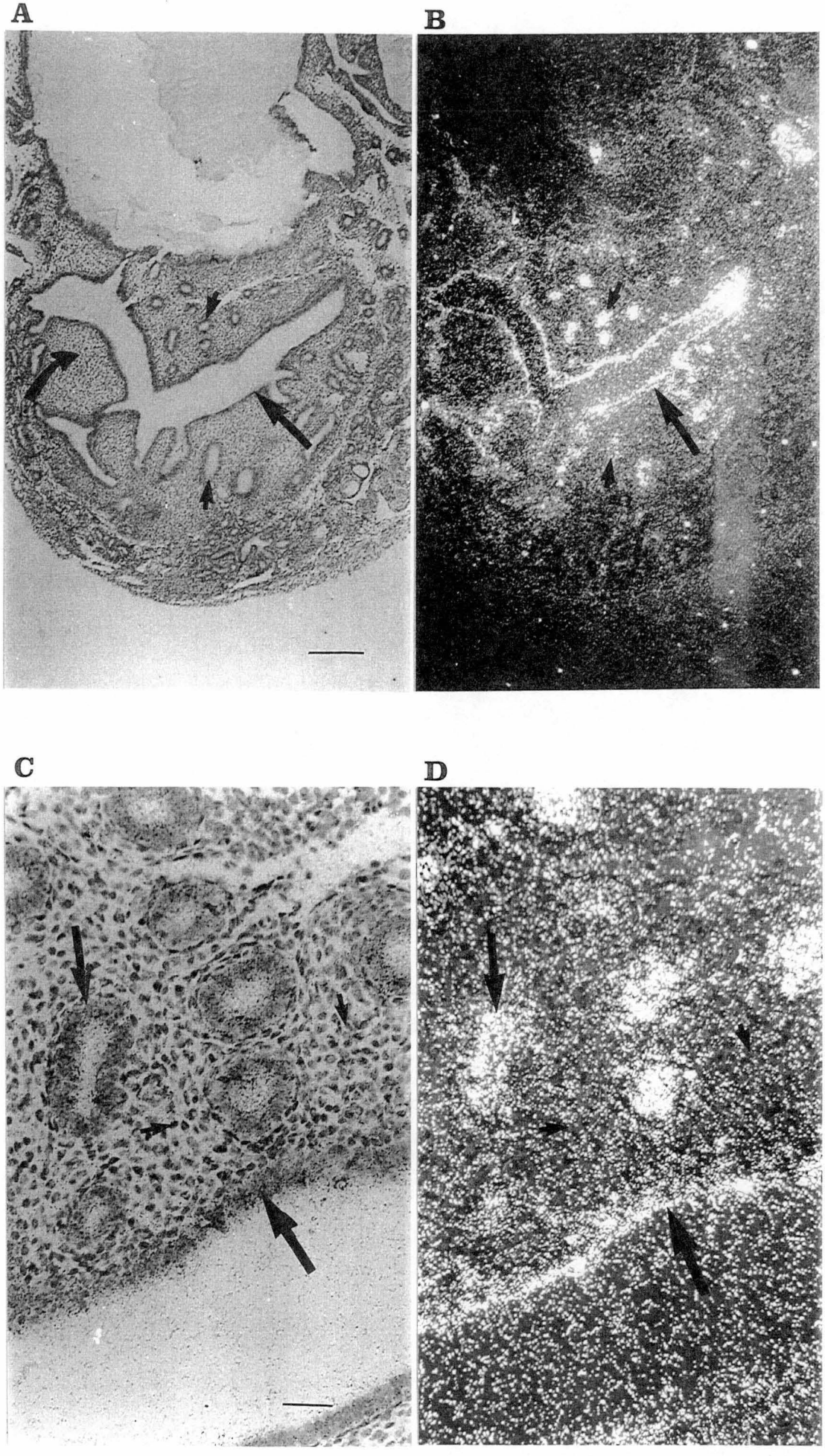
The dorsal surface of the mammalian tongue is predominantly keratinized, stratified, squamous epithelium (Strete, 1995). A transverse section through the opossum tongue (Figs. 4.14-15) shows the compound filiform papillae characteristic of the lingual dorsum of marsupials (Poulton, 1883; Sonntag, 1924). These papillae occur only in the Metatheria and their function is unclear (Krause & Cutts, 1982). They are covered with extensively keratinized epithelium which forms fringe-like projections about the apex of each papilla, and each papilla has a core of vascular connective tissue intruding from the underlying lamina propria (Fig. 4.14-15). Diagrammatic representation of the structure of the mammalian dorsal lingual epithelium is given in Diagram 4.2 (page 95).

Expression of MT mRNA in the opossum tongue co-localises with the lower layers of epithelial cells and the lamina propria (Figs. 4.14, 4.16). The most intense signal is located in the basal and suprabasal layers, and possibly the *stratum spinosum*, of the opossum dorsal epithelium, though isotopic scatter and the intensity of the signal makes precise identification of the labelled cells difficult (Figs. 4.14, 4.15). Metallothionein expression in the epithelium decreases with distance from the basal layers, and granular and surface layers show relatively little signal. Signal appears to extend into the lamina propria below the epithelium, even when the effects of isotopic scatter are taken into account. MT mRNA is most highly expressed on the superior surfaces of the

Fig. 4.13 SALIVARY GLAND

Autoradiographs of transverse section of the opossum submandibular salivary gland. Paraffin sections (0.7μm) were hybridised with ^{32}P-labelled MdMT cDNA and stained with haematoxylin.

A. Salivary gland at low magnification shows large excretory duct in longitudinal section (arrow) and smaller striated ducts in cross-section (small arrows). Secretory units or acini fill the intervening spaces (curved arrow).
   Bar = 500μm
B. Corresponding dark field shows MT mRNA signal in the ductal epithelium of the excretory (arrow) and striated ducts (small arrows).
C and D. High magnification of submandibular salivary gland shows intense MT mRNA expression localised to the ductal epithelium (arrows). Only background levels of signal are evident in the surrounding secretory units (small arrow).
   Bar = 130μm


A
B
C
D

papillae and slightly less on the side surfaces. The papillae of the central part of the dorsal surface express more transcript than do those towards the edges of the tongue (Fig 4.14A).

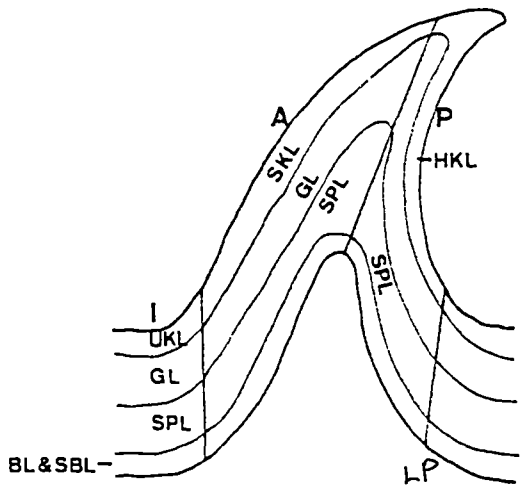


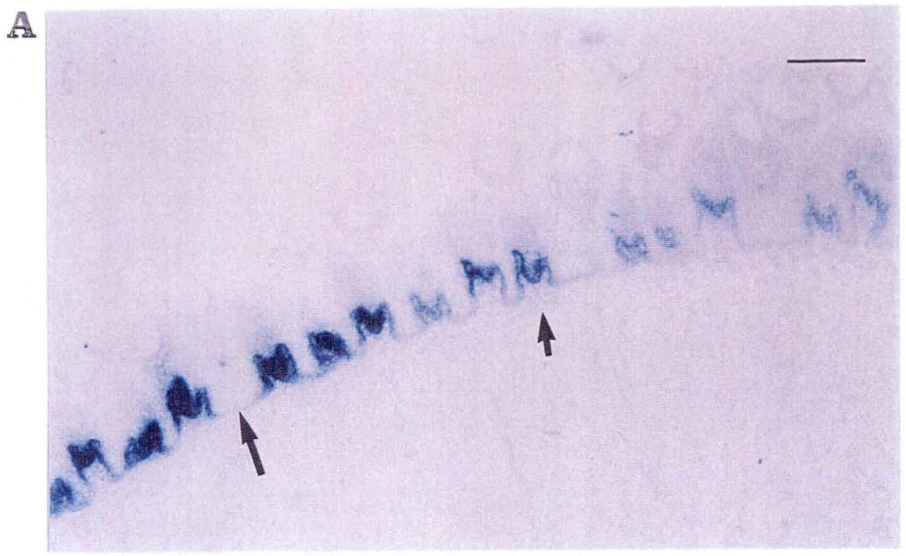
Diagram 4.2: Localisation of cells in each area of the mouse dorsal lingual epithelium. A - the anterior side of the dorsal filiform papilla; P - posterior side of the filiform papilla; I - interpapillary epithelium; LP - lamina propria; BL & SBL - basal and suprabasal epithelium; SPL - spinal layer (*stratum spinosum*); GL - granular layer; UKL - unkeratinized surface layer; SKL - soft-keratinised layer; HKL - hard keratinised layer. Mouse MT1 occurs in the BL & SBL of the anterior papillary epithelium; MT4 in the SPL of the anterior papillary epithelium. Adapted from Iwasaki & Miyata, 1989.

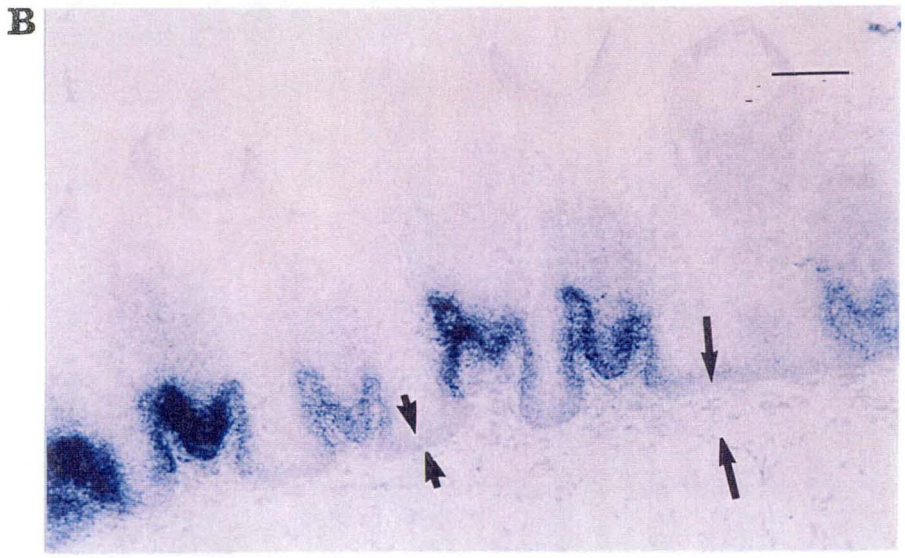
The ventral surface of the opossum tongue is shown in Fig. 4.16 and, like all mammals, has no papillae. MT transcript localizes markedly to the lamina propria as well as to the basal layers of the epithelium. Both regions show similar levels of expression. MT mRNA expression is significantly lower in the basal layers of the ventral epithelium than in those of the dorsal epithelium. Above the suprabasal epithelium, signal diminishes abruptly, and no signal is evident in the cells of the surface mucous membrane, which in eutherian mammals consists of non-keratinised, stratified squamous epithelium (Strete, 1995).

Fig. 4.14 TONGUE

Autoradiograph of a transverse section of the opossum tongue. Paraffin section (0.7μm) was hybridised with ^{32}P-labelled MdMT cDNA and stained with haematoxylin.

A. The dorsal surface of the opossum tongue is covered in keratinised stratified squamous epithelium. Arrows indicate the base of the lingual epithelium. Beneath the epithelium lies a layer of connective tissue, the lamina propia (small arrows indicate the base of the lamina propia). Raised compound filiform papillae occur on the dorsal surface of the tongue, with fringelike projections above the apex of each papilla. Projections of the lamina propia form a core within each papilla. Regions of intense MT mRNA expression (black silver grains) occur in the basal cells of the lingual epithelium. Some papillae demonstrate higher levels of MT expression than others and expression can be seen to diminish from the centre of the tongue (left) towards the edges of the tongue (right).
   Bar = 500μm
B. Higher magnification of the dorsal tongue surface shows more clearly the stratification of the lingual epithelium. Larger arrows indicate the upper and lower surfaces of the lamina propia. MT mRNA expression is extremely strong in the basal and suprabasal cell layer of the epithelium (between small arrows) across the whole dorsal surface of the tongue. Greatest mRNA expression occurs at the upper surface of the papillae, less on the side surfaces and least at the interpapillary epithelium.
   Bar = 250μm
C. Corresponding dark field exposure shows the extreme intensity of the signal at the dorsal lingual surface, and the high level signal in the basal and suprabasal cell layers (between small arrows). The signal in the lamina propia (between larger arrows) is less strong.


A


B

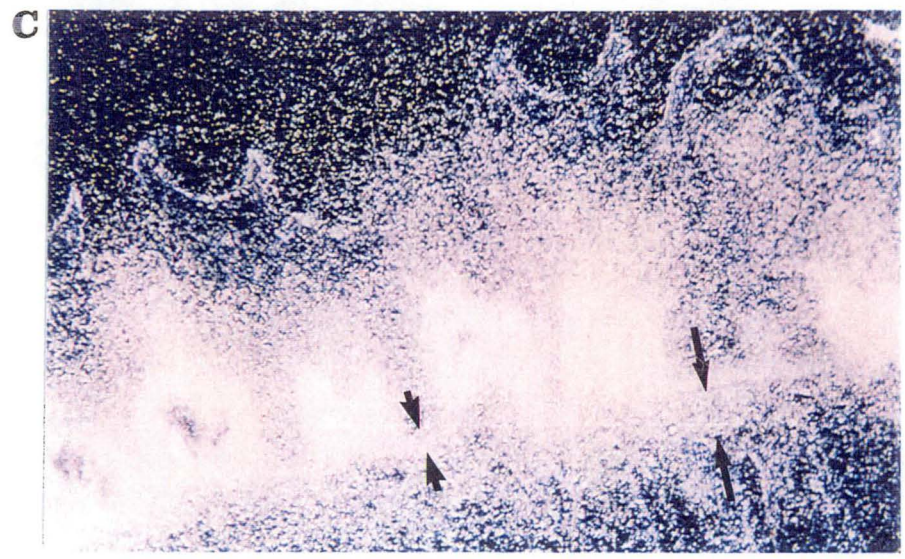

C

Fig. 4.15 TONGUE

Autoradiograph of a transverse section of the opossum tongue. Paraffin section (0.7μm) was hybridised with ^{32}P-labelled MdMT cDNA and stained with haematoxylin.

A. Dorsal surface of the tongue at high magnification, showing the lamina propia (delimited by large arrows); basal and suprabasal epithelium (delimited by small arrows); granular layer (g). Intense deposition of silver grains (black) indicates high levels of expression of MT mRNA in the basal and suprabasal cell layers, extending over the papillae. Signal diminishes abruptly at the interface with the overlying granular layer. Signal in these layers can be attributed predominantly to isotopic scatter.
   Bar = 125μm
B. Corresponding dark field shows such intense signal that much detail is lost with the scatter accompanying ^{32}P detection. Comparison of signal in the granular epithelial layers and inthe lamina propia suggests that not all of the latter can be attributed to the effects of scatter.
C. A single papilla is shown on the right, in cross section through the core of connective tissue (small arrow). A strongly labelled circle of basal epithelium is shown (large arrow).
   Bar = 125μm
D. The corresponding dark field exposure emphasises the distribution of MT mRNA in the basal layers of the epithelium (arrows) around the lamina propia core of the papilla.

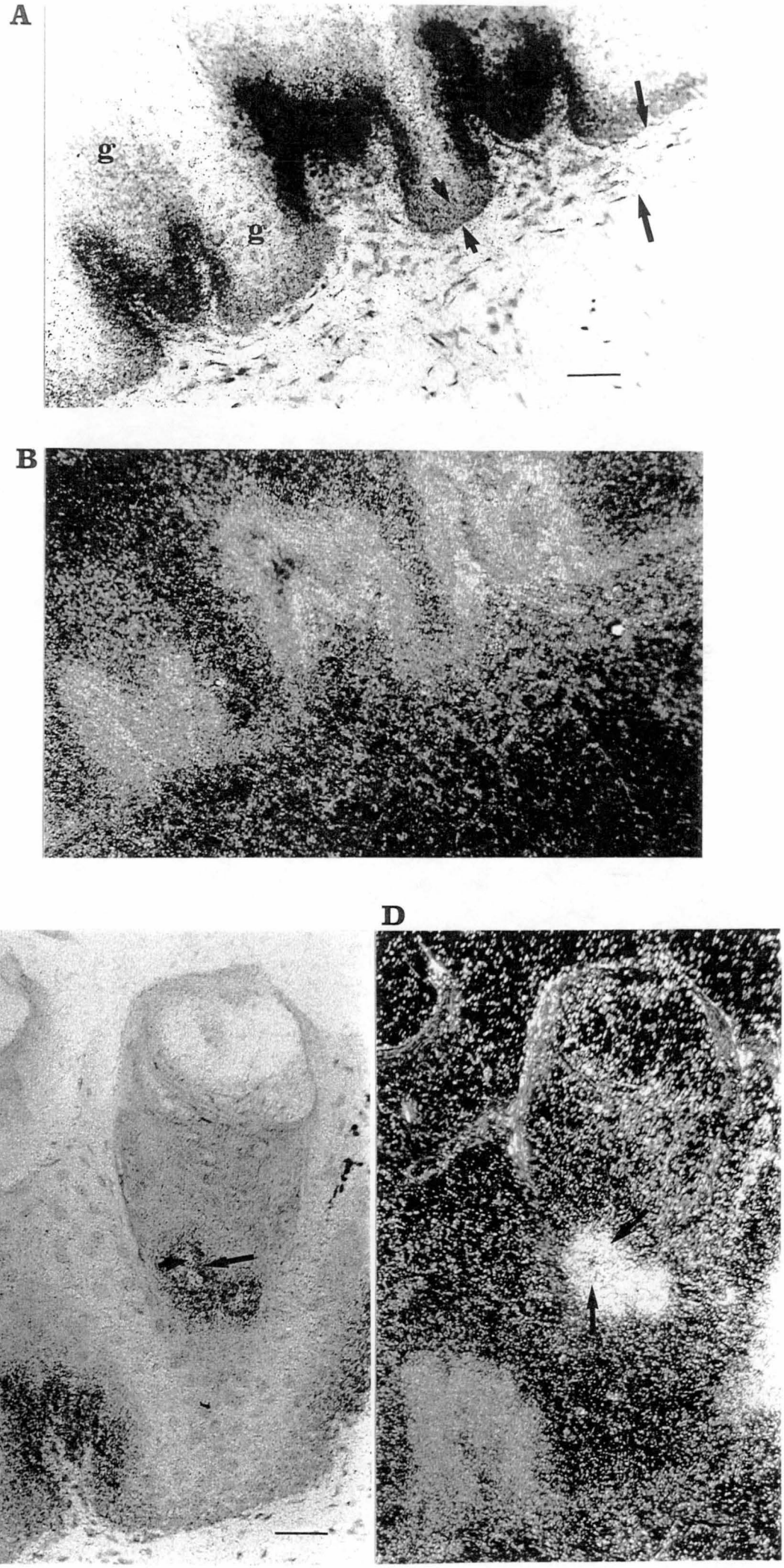

A
g
g
B
C
D

Fig. 4.16 TONGUE

Autoradiograph of a transverse section of the opossum tongue. Paraffin section (0.7μm) was hybridised with ^{32}P-labelled MdMT cDNA and stained with haematoxylin.

A. Ventral surface of the opossum tongue, showing absence of papillae. Lingual salivary glands (curved arrow) occur within the musculature of the tongue. The lamina propia (delimited by small arrows) underlies the ventral lingual epithelium (delimited by large arrows).
   Bar = 500μm.
B. Corresponding dark field exposure. Localisation of MT mRNA within the basal and suprabasal cell layers is not evident at this magnification (large arrow). The lamina propia is strongly positive for MT mRNA (small arrows). This region is shown at higher magnification in C and D. Some parts of the salivary gland system are very strongly positive for metallothionein transcript (curved arrow), though others are not (reflex arrow).
C. Ventral surface of the tongue at higher magnification showing the stratified squamous epithelium (between curved arrows), the basal and suprabasal epithelial layers (between small arrows) and the lamina propia (between large arrows).
   Bar = 125 μm.
D. Corresponding dark field exposure shows intense signal in the suprabasal and basal layers of the epithelium (small arrows) and also in the connective tissue of the lamina propia (large arrows).

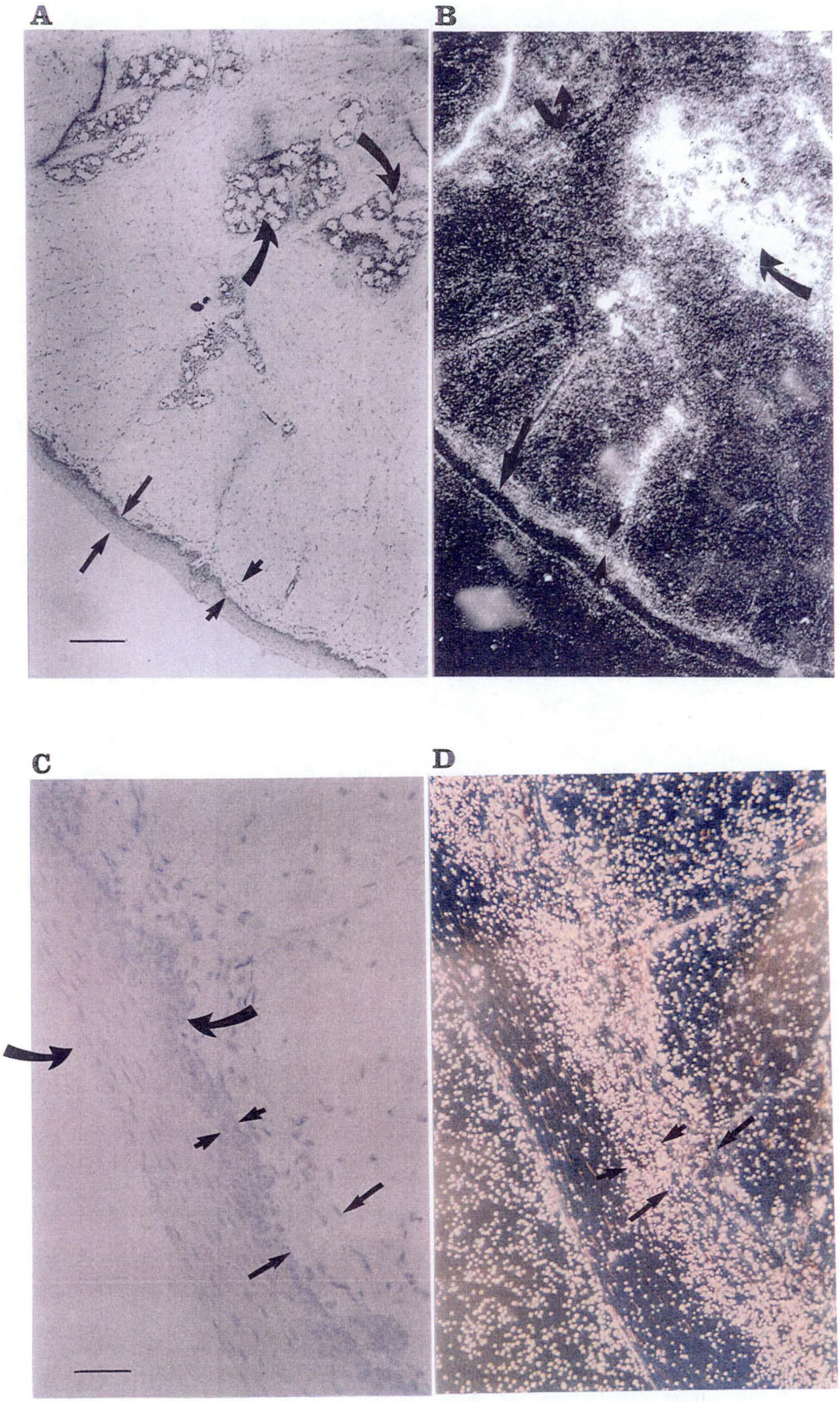
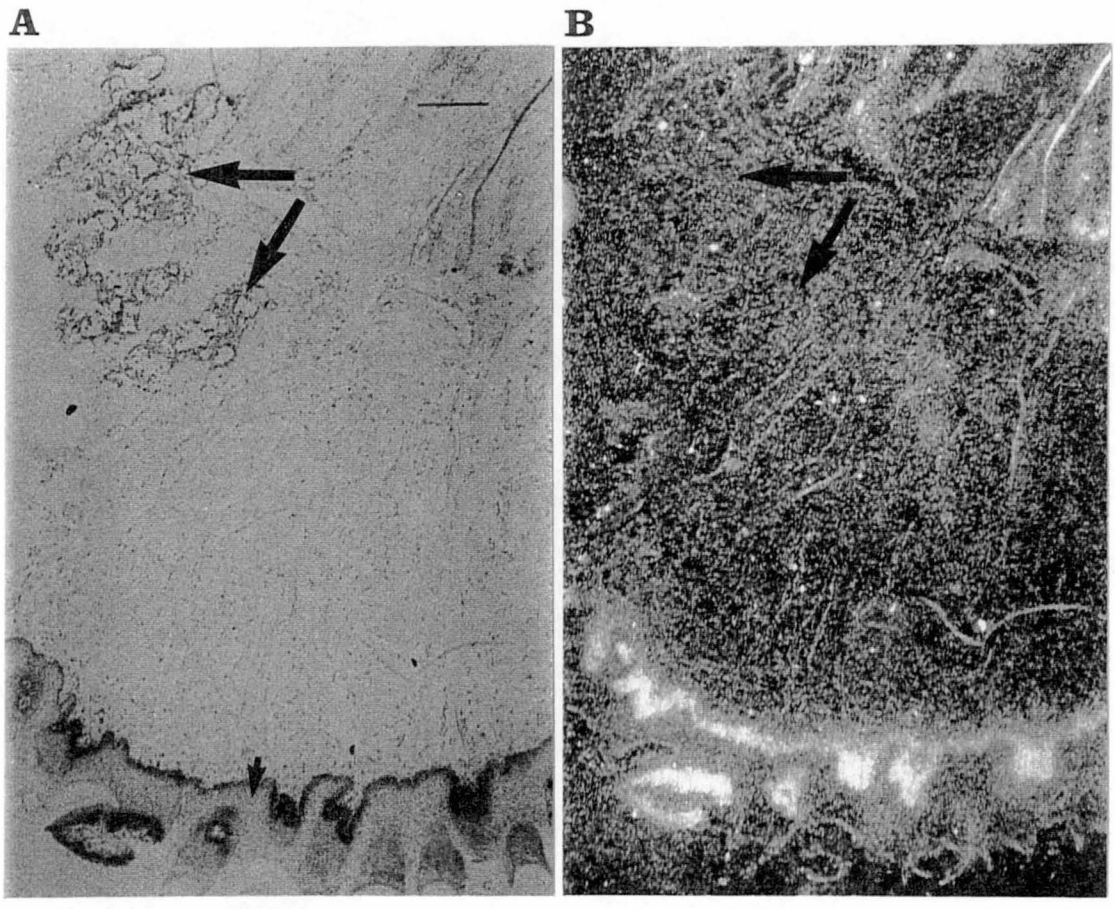

A
B
C
D

Fig. 4.17 TONGUE

Autoradiograph of a transverse section of the opossum tongue. Paraffin section (0.7μm) was hybridised with ^{32}P-labelled MdMT cDNA and stained with haematoxylin.

A. Part of the lingual salivary gland system (large arrows). Dorsal epithelium is indicated (small arrow).
   Bar = 250 μm.
B. Corresponding dark field indicates these salivary gland tissues are MT-negative (arrows).


A
B

MT transcript is also expressed in the lingual salivary glands (Fig. 4.16A, B). Signal is intense within this part of the duct system (curved arrow). Patterns of MT mRNA expression observed here correspond to those shown in the submandibular salivary gland of Fig. 4.13. Some parts of the glandular tree, however, do not show this intense MT mRNA-positive staining (reflex arrow) and both MT-positive and -negative salivary gland tissue is found throughout the tongue (Figs. 4.16 and 4.17).

4.3 DISCUSSION

4.3.1 MT mRNA expression in adult tissues

The *MT* genes of the opossum are not transcribed equivalently in all tissues but display a distinct profile across tissues (Fig. 4.4). However, mammalian metallothionein expression is subject to regulation at many levels, including the translational (Lehman-McKeeman *et al.*, 1988b; Vasconcelos *et al.*, 1996), and instances have been documented where there is little or no correspondence between the incidence of MT transcript and MT protein (Paynter *et al.*, 1990; Misra *et al.*, 1997; Scudiero *et al.*, 1997). Therefore, levels of MT mRNA in opossum tissues cannot be presumed to reflect the protein content of these tissues. Prior reports of the relative expression of metallothionein between tissues in other mammals have been based on an assessment of protein content, which makes any comparison with opossum data tenuous. The trends, however, correspond reasonably well between opossum transcript levels reported here and the MT protein content documented for other mammals, in the kidney, testis, spleen, heart, lung and muscle.

4.3.1.1 Liver and kidney

MT mRNA is most strongly expressed in both the opossum liver and kidney, of the tissues examined. Immunohistochemical localisation of MT 1 and 2 in the dog indicates equally high levels in the adult kidney and liver (Shimada *et al.*, 1997). Levels documented for the adult human liver are much higher than those of the kidney, though variation between samples is also high (Nakajima & Suzuki, 1995); the opposite relationship has also been reported (Heilmaier *et al.*, 1987). The principal discrepancies in tissue-specific measures of MT involve the

proportions of hepatic and renal MT. Levels of hepatic MT vary across species of eutherian mammals and with detection techniques: high levels in mouse liver by Cd/haemoglobin assay (Iszard *et al.*, 1995); medium levels in rats using Cd-saturation assay (Chen & Ganther, 1975); and low levels in rat liver detected with RIA (Nolan & Shaikh, 1986; Mehra & Bremner, 1987). Species-specific differences in levels of hepatic metallothionein have been demonstrated to two orders of magnitude (Henry *et al.*, 1994), and variation in the expression of MT across tissues types also noted between species (Zelazowski & Piotrowski, 1977). The extreme sensitivity of hepatic metallothioneins to induction may contribute to differences in levels of MT recorded in the liver. Constitutive levels of hepatic MT in normal sheep has been found to correspond to levels in cultured fibroblasts maximally induced with heavy metals (Peterson & Mercer, 1988). Insofar as much variability exists in the distribution of MT in the liver and kidney of the eutheria mammal, the expression profile of the opossum metallothioneins is in accord with these.

4.3.1.2 Brain

MT mRNA in the opossum brain is high, but well below levels found in liver, kidney, tongue and salivary gland. Constitutive levels of MT1 in the mouse brain were higher than hepatic levels of MT and showed less variability between animals (Zheng *et al.*, 1995). A 20-fold difference exists between levels of MT1 in the mouse and rat brain (Choudhuri *et al.*, 1993).

4.3.1.3 Tongue

Extremely high levels of basal *MT* transcription are shown here in the opossum tongue. The relative concentration of MT or the corresponding mRNA has not been described for the tongue of the eutherian mammal. Both MT1 and MT4 have been shown to be transcribed in the mouse tongue epithelium (Quaife *et al.*, 1994), but the extent of this expression has not been documented. Although MT3 was initially described as a brain-specific isoform of metallothionein (Uchida *et al.*, 1991; Tsuji *et al.*, 1992), recent studies report the occurrence of MT3 mRNA in the rat tongue (Moffatt & Seguin, 1998). Tongue MT3 mRNA occurs at one fifth the level of brain MT3 mRNA in the rat. These findings distinguish tongue as one of very few tissues in the eutherian mammal

known to transcribe the complete metallothionein locus. Investigation of the prevalence of the phenomenon among the vertebrates is therefore warranted.

4.3.1.4 Salivary gland

High levels of MT mRNA are also demonstrated in the opossum salivary gland. *M. domestica* is noted for the abundance of its saliva, attributable in part to its role in thermoregulation (Higginbotham & Koon, 1955; Wilborn & Shackleford, 1969). Data are not available on the relative levels of *MT* expression in the eutherian salivary gland. Whether high levels in this tissue are typical of mammals or correlate with extremes of secretion is yet to be established.

4.3.1.5 Testis

Metallothionein is also transcribed significantly in the opossum testis. This is consistent with what is known of MT expression in the eutherian testis, where MT mRNA is present in spermatocytes and spermatids, but in neither Sertoli cells nor spermatogonia (Tohyama *et al.*, 1994). MT protein, however, is localised to slightly different parts of the testis: to Sertoli and interstitial cells (Danielson *et al.*, 1982a; Nolan & Shaikh, 1986) and to the tubules containing differentiating spermatogenic cells but not spermatozoa (Nishimura *et al.*, 1990). The role of MT in the testis has been postulated to involve metal storage, transport and detoxification (Onosaka & Cherian, 1982), as well as cell proliferation and differentiation (Nishimura *et al*, 1990).

4.3.1.6 Lung

Levels of metallothionein mRNA in the opossum lung are low, though higher than both heart and skeletal muscle. MT has been demonstrated immunohistochemically in the normal human lung in activated macrophages, in the pleural endothelial cells and in the basal cells of the bronchial epithelium (Courtade *et al.*, 1998). *In situ* hybridization demonstrates high levels of MT transcript in the bronchial epithelium (Gilks *et al.*, 1998).

4.3.1.7 Spleen, heart, muscle

The opossum spleen displays very low levels of MT mRNA. In several mammalian species (rat, cow, mouse, rabbit), MT content of the spleen is very high, comparable to the content of kidney . In the pig, however, levels of MT in the spleen are low. Low levels of MT mRNA in the opossum heart and skeletal

muscle are consistent with the findings for other species (Zelazowski & Piotrowski, 1977)

4.3.2 Expression of different MT transcripts in tissues

The transcription of all opossum metallothionein mRNAs is highest in the liver, with varying levels in other tissues examined. Each sequence is transcribed in the brain, but at a concentration well below that of the liver. (Extremely low levels of MTx5 are detected in the brain. This transcript was, however, isolated as two clones from the opossum brain cDNA library, confirming its expression in this tissue.) The expression profiles of transcripts B1, B3 and L2 are very similar to that of the generalised distribution of opossum MT mRNA (Figs. 4.1, 4.2). Transcripts L3 and x5 display a more restricted distribution. It may be significant that the former three transcripts share sequence characteristics in the 5' and 3' untranslated regions that are less well conserved in the latter two transcripts, as described in Chapter 3 (3.3.6).

It is noteworthy that the high level of MT mRNA expression in the kidney has no parallel in the expression of any of the individual transcripts, which may suggest the existence of a further transcript, not yet isolated. These results demonstrate that the multiple *MT* genes of the opossum are transcribed in many non-hepatic tissues, without experimental induction.

4.3.3 Developmental expression of MT mRNA

4.3.3.1 Liver

There is a steady increase in hepatic metallothionein mRNA expression in the opossum from birth to adult. This differs greatly from eutherian ontogenic expression patterns, where fetal or perinatal hepatic MT expression is considerably higher than that of the adult (Bell, 1979; Wong & Klaassen, 1979; Waalkes & Bell, 1980; Bakka & Webb, 1981). The occurrence of a pre-natal peak of hepatic MT expression in the opossum, comparable to that of the eutherian fetus, is unlikely. Placentation is short-lived in the opossum and superficial (Harder *et al.*, 1993; Mate *et al.*, 1994), and the liver is extremely underdeveloped prior to birth (Krause & Cutts, 1984; 1986).

Metabolic differences exist between the marsupial and eutherian neonates, that arise from differences in their maturity. The metabolic rate of mammals is determined by their surface-to-volume ratio, as dictated by the exigencies of thermoregulation (Kleiber, 1972). The fetal mammal, however, has a metabolic rate determined by the metabolic rate of the mother, itself dictated by the mother's size (Wieser, 1984). At birth, the eutherian neonate undergoes a metabolic switch from the maternal metabolic rate to one consistent with its small body size. Metabolic rate increases rapidly with concomitant escalation of oxygen consumption (Hill & Rahimtulla, 1965). The marsupial neonate, on the other hand, does not make this metabolic switch, and continues for some time at a metabolic state comparable to that *in utero* (Singer, 1995a; 1995b). The neonatal marsupial is, therefore, a true poikilotherm (Petajan & Morrison, 1962). Furthermore, low oxygen consumption is obligatory for the newborn opossum due to physiological constraints attendant upon its immaturity, including incomplete tissue vascularisation, especially of the lungs and brain. The mechanisms which allow the marsupial to combine low metabolic rate, thermostability and growth efficiency are not understood (Singer, 1998). A role for MT in the mediation of oxidative stress has been suggested (Thornalley & Vasak, 1985). Because the marsupial neonate is not exposed to the full physiological impact of oxygen at birth as is the eutherian neonate, the role of metallothionein, and therefore its expression, may be significantly different. The rise in MT expression in *Monodelphis* may correspond to increasing oxygen consumption with the acquisition of endothermy, which should occur over roughly this time course if it parallels the progression to weaning in the opossum, as is the case for other marsupials (Tyndale-Biscoe & Janssens, 1988).

Although the liver of the eutherian mammal is formed quite early in embryonic life, birth signifies an abrupt secondment into various homeostatic functions previously perfomed by the placenta and maternal liver. Because there is little dependence of the marsupial fetus on placentation (Harder *et al.*, 1993; Mate *et al.*, 1994) and most development proceeds postnatally, the marsupial liver adopts these functions more gradually. In this way too, there is no discontinuity in marsupial liver function at the time of birth. The peak of MT accumulation in the gestational or perinatal eutherian liver may be involved in

accommodating a sudden recruitment of the organ into its various roles, with a requirement for the management of metal ions for the metabolic activity associated with growth. Metal regulation in the opossum liver may evolve more gradually, in accord with the liver's acquisition of function, and MT accumulation may be correspondingly incremental.

4.3.3.2 Brain

MT mRNA is present in the opossum brain at birth. The brain of the newborn possum is developmentally comparable to that of the embryonic rat at 13 days post-conception (E13) (Saunders *et al.*, 1989). Data is not available for MT1 mRNA expression in the rat embryo, but MT1 and MT3 transcripts are both detected by solution hybridisation in the head of the embryonic mouse at E16.5 (Masters *et al.*, 1994b), though not until P14 by northern analysis (Choudhuri *et al.*, 1996). MT1 transcripts increase steadily in the mouse to adult levels around the second week of life (Masters *et al.*, 1994b). Immunohistochemistry first localises MT1/2 in the human fetal brain at 35 weeks (Suzuki *et al.*, 1994). The brain of the neonatal opossum is equivalent to the human fetal brain at 6 weeks post-conception (Saunders *et al.*, 1989), so *MT* expression occurs developmentally much later in the human fetus than in the opossum. The neonatal opossum brain compares developmentally with the E26-30 sheep brain (Saunders *et al.*, 1989). MT1 mRNA is first detected by northern blotting in the sheep brain at E72 (Holloway *et al.*, 1997a), when the embryonic brain is considerably more mature than that of the neonatal opossum. Adult levels are reached in the sheep before birth, at E115 (sheep gestation is 150 days).

MT1 and MT3 have very similar, though not identical developmental profiles in the eutherian brain (Kobayashi *et al.*, 1993; Masters *et al.*, 1994a; Holloway, 1996; Ono & Cherian, 1998). Choudhuri *et al.* (1996) suggest that adult levels of MT1 and MT3 mRNA are not attained until P60 in the mouse. However, adult levels of MT1 and MT3 mRNA have been recorded in the second week of life in the brains of both the rat (Kobayashi *et al.*, 1993) and mouse (Masters *et al.*, 1994b). Rat MT1 (Waalkes & Klaassen, 1984) and mouse MT3 (Ono & Cherian, 1998) have been detected at adult levels at P21. Adult

levels of transcript are not detected in the opossum till after P40. Therefore, in summary, metallothionein mRNA appears developmentally earlier in the *Monodelphis* brain than in the eutherian, and takes longer to reach adult levels. This is also true of other cerebral proteins of *Monodelphis* (Dore *et al.*, 1990).

The rise of MT mRNA in the opossum brain from P10 parallels the postnatal expression of both MT1 and MT3 in the rodent brain relative to the time of birth, rather than to the developmental stage of the neonate. The period between P9 and P60 is critical for brain development in the mouse, and corresponds to the rise in expression of MT1 and MT2 (Ono & Cherian, 1998). Similarly, most cortical development and neurogenesis occur in the opossum from P7 to P60 (Saunders *et al.*, 1989). Metallothionein transcript begins to rise gradually in the opossum brain at P10 or before, if the sensitivity limitations of northern blotting are taken into account. This is coincident with cortical plate development, which commences at P7 (Saunders *et al*, 1989). Astrocyte maturation occurs during the period P1-P25 (Elmquist *et al.*, 1994) and most neurogenesis occurs in the second and third weeks after birth (Clark & Smith, 1993), though differences in brain regions exist (Harman, 1997). MT transcript levels rise sharply after P25 in the opossum, at which time astrocyte maturation and neurogenesis is largely complete.

Too little is known at present of the structure and development of the opossum brain to make definitive statements about its relation to MT expression. However, it is significant that the marked differences in hepatic MT expression that occur between eutherian and opossum neonates have no counterpart in the developing brain.

4.3.4 MT mRNA expression at the cellular level

4.3.4.1 Liver

The homogeneity of MT mRNA signal displayed in the opossum liver (Fig. 4.7) is consistent with the distribution of MT in hepatocytes, and corresponds to the occurrence of hepatic MT1 and MT2 in eutherian mammals. Metallothionein has been localised immunohistochemically to the cytoplasm of all hepatocytes in the uninduced rat liver, though to few hepatocyte nuclei

(Danielson *et al.*, 1982b). Endogenous levels of MT are higher in Kupffer cells (specialised macrophages) and hepatic endothelial cells, than in hepatic parenchyma cells (McKim *et al.*, 1992), but the latter constitute the bulk of hepatic tissue. MT is also detected in sinusoids and canaliculi (Elmes *et al.*, 1987; Shimada *et al.*, 1997).

4.3.4.2 Kidney

No transcription of metallothionein was detected in the glomeruli of the opossum kidney. High levels of MT mRNA, however, occur throughout the cortex and medulla, and are specifically demonstrable in the epithelia of the various tubules that aggregate to form these tissues. These findings are in accord with the expression of *MT1* and *MT2* in the eutherian kidney.

MT has been detected immunohistochemically in the epithelial cells of the renal tubules that comprise the nephron (Danielson *et al.*, 1982b; Diagram 5.1). Many (but not all) cells of the proximal tubules are MT-positive in the rat (Danielson *et al.*, 1982b; Nartey *et al.*, 1987) and biosynthesis and degradation of Cu-MT has been shown to recur cyclically in the renal proximal tubules (Kurasaki *et al.*, 1998). MT has been detected exclusively in the proximal tubules of the human kidney (Mididoddi *et al.*, 1996), and predominantly in the proximal tubules of the dog (Shimada *et al.*, 1997). MT has been localised to the distal tubules of the renal cortex of the rat (Banerjee *et al.*, 1982; Elmes *et al.*, 1987). To this localisation has been attributed the relative invulnerability of the distal tubules to the nephrotoxic effects of cadmium, which act principally at the proximal convoluted tubules (Kendall *et al.*, 1983). The squamous epithelial cells of the glomeruli, vascular and connective tissue are negative for metallothionein (Danielson *et al.*, 1982a; 1982b; Shimada *et al.*, 1997). The medullary collecting ducts are MT-positive (Danielson *et al.*, 1982b; Banerjee *et al.*, 1982; Elmes *et al.*, 1987). MT mRNA expression has been shown in the cortex and outer stripe of the outer medulla (Diagram 4.1) and this transcription is elevated during compensatory renal growth after uninephrectomy (Zalups *et al.*, 1995).

4.3.4.3 Brain

The most striking expression of MT mRNA in the marsupial forebrain is seen in the ependymal cells of the lateral and third ventricles. Ependymal cells are singly stratified cuboidal cells lining the cerebral ventricular surfaces, which form part of the cerebrospinal fluid (CSF)-brain barrier in mammals (Bruni *et al.*, 1985). Strong immunohistochemical staining for MTs 1 and 2 has been demonstrated in the ependyma in the mouse (Nishimura *et al.*, 1992), the rat (Young *et al.*, 1991; Nakajima & Suzuki, 1995) and the human (Suzuki *et al.*, 1994). Immunostaining has shown MT in both the cytoplasm and nuclei of ependymal cells (Nishimura *et al.*, 1992). *In situ* hybridization indicates that MT1 mRNA is expressed in the mouse ependymal epithelia (Choudhuri *et al.*, 1995), although some reports indicate that MT1 (Nishimura *et al.*, 1992) and MT1 mRNA (Itano *et al.*, 1991) appear in the rat ependymal cells only in response to intraperitoneal lipopolysaccharide (LPS). The presence (Masters *et al.*, 1994b) and absence (Choudhuri *et al.*, 1995) of MT3 transcript has been reported in these cells using *in situ* hybridization, and MT3 has been detected immunohistochemically with antiserum to a synthetic polypeptide specific for rat MT3 (Yamada *et al.*, 1996). It has been suggested that the localisation of MT to the ependymal epithelia implicates these cells in the regional restriction of heavy metals in the brain (Nishimura *et al.*, 1992; Gasull *et al.*, 1994). The expression of MT has also been linked to cell growth in the epithelia of the ependyma and the choroid plexus (Nishimura *et al.*, 1992). However, although ependymal epithelial cells are very active in proliferation in fetal and neonatal rats, they are not proliferative in the adult (Bruni *et al.*, 1985).

The choroid plexus of the opossum shows some MT transcript. This is verified by relatively high levels of MT mRNA in the adult choroid plexus demonstrated on the northern blot of Fig. 4.5. MT is not detected in the choroid plexus of the mouse (Nishimura *et al.*, 1992) and MT1 mRNA detected only after treatment with LPS (Choudhuri *et al.*, 1995; Zheng *et al.*, 1995). However, MT1/2 immunoreactivity has been demonstrated in the choroid plexus epithelium of the adult rat (Penkowa & Moos, 1995). MT1/2 have been detected immunohistochemically in the cytoplasm and nuclei of both the human choroid plexus and ependymal epithelia (Nakajima & Suzuki, 1995), though its absence

had previously been reported in both these tissues (Blaauwegers *et al.*, 1993). Constitutive expression of MT3 mRNA has been detected in the choroid plexus of the mouse (Masters *et al.*, 1994b), and MT3 protein detected immunohistochemically (Yamada *et al.*, 1996). Other workers, on the contrary, failed to detect MT3 mRNA in the mouse choroid plexus (Choudhuri *et al.*, 1995). Constitutive expression of MT1, MT2 and MT3 mRNA in the sheep choroid plexus has been demonstrated by northern analysis (Holloway, 1996; Holloway *et al.*, 1997a). The choroid plexus lies outside the blood-brain barrier (Cserr & Bundgaard, 1984). It is formed from an extension of the ependymal epithelium (Sarnat, 1998) and functions in the secretion of cerebrospinal fluid (Milhorat,1976). It constitutes part of the barrier between the blood and the CSF (Bruni *et al.*, 1985).

Capillaries within the periventricular grey matter of the third ventricle show strong signal for MT mRNA, inferred to arise in the vascular endothelium. It is the neurovascular endothelium that constitutes the blood-brain barrier. Intense signal about these capillaries is attributed to glial cells which encircle cerebral capillaries (Goldstein, 1988), and which have been shown to support the blood-brain barrier phenotype (Janzer & Raff, 1987). Metallothionein 1/2 have been demonstrated immunohistochemically in circumcapillary astrocytes in the developing and adult sheep brain (Holloway, 1996). High levels of MT3 have also been identified in astrocytic end-feet encircling blood vessels in the rat, using a polyclonal antibody against rat MT3 (Yamada *et al.*, 1996). These instances have been cited as corroboration of a protective role for metallothionein against heavy metal toxicity at the blood-brain barrier.

MT transcripts are expressed throughout the opossum hippocampus, with intensified expression in the granular layers of the dentate gyrus (Figs. 4.10, 4.12). Both MT1 and MT3 are expressed in the hippocampus, identified immunohistochemically (Young *et al.*, 1991; Yamada *et al.*, 1996) and by the analysis of transcription products (Masters *et al.*, 1994b; Choudhuri *et al.*, 1995). Mouse MT3 transcript is demonstrable in the granular cell layer of the dentate gyrus of the hippocampus by *in situ* hybridization (Masters *et al.*, 1994b; Choudhuri *et al.*, 1995; Zheng *et al.*, 1995), with weaker signal over the fibre-rich regions of the molecular layer (Masters *et al.*, 1994b). MT1 mRNA, on the

other hand, is not always detectable in the hippocampus by *in situ* hybridisation (Masters *et al.*, 1994b; Zheng *et al.*, 1995; Choudhuri *et al.*, 1995). The cerebral cortex, and in particular the hippocampus, contains the highest concentration of zinc in the brain (Frederickson *et al.*, 1983) and MT3 mRNA has been shown to co-localise with zinc-sequestering neurons in this region (Masters *et al.*, 1994b). Cerebral copper accumulation is also highest in the hippocampus (Ono *et al.*, 1997)

Eutherian MT1, MT2 and MT3 transcripts and protein are expressed throughout the various regions of the brain at similar levels (Masters *et al.*, 1994b; Choudhuri *et al.*, 1995). However, MT1/2 and MT3 mRNA have been distinguished in characteristic cell types within these regions (Masters *et al.*, 1994b; Zheng *et al.*, 1995; Choudhuri *et al.*, 1995). Both MT1 (Choudhuri *et al.*, 1995; Zheng *et al.*, 1995) and MT3 (Masters *et al.*, 1994b) transcripts have been demonstrated by *in situ* hybridisation in the ependymal epithelium of the mouse, and also in the choroid plexus. The preferential localisation of MT3 mRNA to the granular layer of the hippocampal dentate gyrus is clearly evident with *in situ* hybridisation techniques (Choudhuri *et al.*, 1995; Zheng *et al.*, 1995). Although MT1 mRNA is abundant in the hippocampus, as demonstrated in northern analysis (Masters *et al.*, 1994b; Holloway 1996; Holloway *et al.*, 1997a), its dispersed distribution means it is not always detected by *in situ* hybridisation (Masters *et al.*, 1994b; Zheng *et al.*, 1995). Choudhuri *et al.* (1995) alone report detection of a diffuse level of MT1 mRNA in the mouse hippocampus by *in situ* hybridisation, but the data are not shown, suggesting a very low and perhaps photographically irreproducible signal. MT transcripts of the opossum have been localised to the regions within the hippocampus where only MT3 mRNA is readily demonstrable in the eutherian brain. It is not possible with the data presented here to correlate the individual opossum MT transcripts with particular cell types. However, such a correlation would be useful. If any or all of the opossum MT mRNAs described here (Chapter 3) are those expressed in the granular layer of the dentate gyrus, they may have a functional correlation with eutherian MT3. If none of the documented opossum MT mRNA localise to the granular layer of the dentate gyrus, these *in situ* hybridization results suggest that

a further as-yet-undefined putative MT3 homologue may be expressed in this region.

4.3.4.5 Salivary gland

MT mRNA is expressed at high levels in the excretory and striated ducts of the opossum submandibular gland. Excretory and striated ducts have much in common and it has been suggested that, in marsupials, a sharp distinction beween different parts of the salivary ductal system cannot be made (Young & van Lennep, 1978). Cells of the striated and excretory ducts do not merely form conduits, but contribute to the water (Babkin, 1950) and salt (Wilborn & Shackleford, 1969) balance of saliva, and are presumed to have high metabolic activity (Schneider & Person, 1960).

Metallothionein has been identified in the basal cells of the excretory and striated ducts of the human salivary gland (Sunardi-Widyaputra *et al.*, 1995; Shrestha *et al.*, 1996); and also in myoepithelial cells (van den Oord *et al.*, 1993; Sunardi-Widyaputra *et al.*, 1995), though this has been disputed (Shrestha *et al.*, 1996). The function of the subpopulation of MT-expressing ductal cells located at the non-luminal aspect of the excretory and striated ducts is not clear (Shrestha *et al.*, 1996). Myoepithelial cells, on the other hand, are thought to have a contractile function and lie between the basement membrane and the cells of secretory units and intercalated ducts (Young & van Lennep, 1978). They are especially dense in the opossum submandibular gland, and are implicated in the secretion of copious saliva for which the opossum is noted (Wilborn & Shackleford, 1969). MT mRNA expression was not established in the non-ductal regions of the opossum salivary gland in this work. This would suggest that myoepithelial cells do not express metallothionein in the opossum salivary gland.

4.3.4.4 Tongue

The dorsal surface of the mammalian tongue is predominantly keratinized, stratified, squamous epithelium, while the ventral surface mucous layer is non-keratinized, stratified, squamous epithelium (Strete, 1995). Metallothionein expression in the tongue has been described in the mouse (Quaife *et al.*, 1994). MT1 mRNA (and presumably MT2 mRNA) is expressed in

the basal layer of the dorsal and ventral lingual epithelia, and MT4 mRNA is expressed in the cells of *stratum spinosum* immediately above the basal layer. The presence of MT1 and MT2 has been confirmed immunohistochemically in the human tongue, in the basal and parabasal cells of the lingual epithelium (Sundelin *et al*, 1997). It is suggested that the switch in expression from *MT1* in the basal, proliferative layers to *MT4* in the suprabasal, differentiating layers indicates differing roles for each isoform in the keratinization process (Quaife *et al.*, 1994). On the papillae of the mouse dorsal epithelium, both MT1 and MT4 transcripts are expressed more strongly on the anterior surfaces than on the posterior surfaces (Quaife *et al.*, 1994), reflecting differences in keratin type at these sites (Tobiasch *et al.*, 1992; Dhouailly *et al.*, 1989). No MT mRNA was detected in the lamina propria of the mouse (Quaife *et al.*, 1994).

The papillae of the opossum tongue don't conform morphologically to the filiform papillae of the human or mouse tongue, and a pronounced species variation is recognised in the filiform papillae of mammals (Boshell *et al.*, 1982). Therefore, parallels between the opossum tongue and that of other mammals must be drawn with circumspection. Papillae of the opossum dorsal lingual epithelium are compound filiform, covered in highly keratinised epithelium (Poulton 1883; Sonntag 1924).

Due to the intensity and scatter of the signal, it is not possible to distinguish precisely the cellular location of metallothionein mRNA expression in the opossum tongue. On the dorsal tongue surface, most intense signal is clearly above the lamina propria within the lower layer/s of the epithelium and diminishes quickly towards the epithelial surface. Virtually no signal is evident in the surface layers of the epithelium, corresponding to the keratinized cells of Diagram 4.2. In contrast to the mouse tongue, the tongue of the opossum shows definite signal localised to the lamina propria of the ventral surface which probably has a counterpart on the dorsal surface, masked by the scatter from the very intense signal from the basal epithelial layers. The basal cell layers of the ventral epithelium also express MT mRNA, though at levels only slightly higher than those of the lamina propria, and markedly less than the basal cells of the dorsal epithelium. Because this section of opossum tongue is transverse, it is not possible to distinguish anterior and posterior papillary surfaces which in the

mouse show different levels of metallothionein expression. However, where papillae are transected, giving circular cross-sections of the central core of lamina propria (Fig. 4.15), no difference in the intensity of signal in the circle of basal epithelial cells was seen, indicating a homogeneity of expression on all surfaces of the papilla. In the mouse, the anterior surface of the filiform papillae is formed from cells expressing soft keratins; cells of the posterior surface of the papillae express hard keratins (Dhouailly *et al.*, 1989). MT transcription in the mouse tongue co-localises with the soft-keratinizing cells (Quaife *et al.*, 1994), the only compartment of the mouse dorsal lingual epithelium to display a granular epithelial layer (Tobiasch *et al.*, 1992). The *stratum granulosum* occurs over the whole dorsal epithelium in this region of the opossum tongue, however, and is several layers thick (Krause & Cutts, 1982). A correlation may exist between the extensive granular layer, an associated form of soft-keratinization and the very high level of metallothionein transcript in the opossum tongue.

Expression patterns of the opossum metallothionein mRNA basically support the findings of Quaife *et al.* (1994) but their fuller significance must await analysis of individual opossum MT transcripts, and a better understanding of the fine structure of the opossum tongue. Eighty percent of MT in the mouse tongue occurs as the MT4 isoform, with a concentration calculated at 4.5 million molecules per epithelial cell (Quaife *et al.*, 1994). Whether the metallothionein transcripts detected in the opossum tongue correspond to an MT4 homologue over and above the MT mRNAs already identified for the opossum, or whether the latter fulfil the functional role of mammalian MT4 remains to be determined. Given that the tongue is one of the few sites at which the complete MT locus is transcribed in the rat (Moffatt & Seguin, 1998), further investigation of MT transcript localisation in the opossum tongue is warranted.

4.3.5 Summary

The profile of metallothionein expression in the opossum is fundamentally similar to that of the eutherian metallothioneins 1 and 2. The transcripts described in Chapter 3 are detectable in virtually all tissues examined, with a slightly different distribution peculiar to each transcript. The tissue-

specificity described for isoforms MT3 and MT4 have no parallel in the opossum MT mRNAs.

Cell type-specific expression of the opossum MT transcripts similarly draws strong parallels with the distribution of eutherian MT1 and MT2. Because eutherian MT3 is expressed in regions also expressing MTs 1 and 2, it is difficult to dissect out any correspondence between opossum MT mRNAs and eutherian MT3. The granular layer of the hippocampal dentate gyrus is one of the few tissues in which MT3 mRNA can be detected uniquely, and therefore such a distinction made. MT mRNA in the opossum hippocampus is clearly shown by *in situ* hybridisation, and corresponds to the occurrence of MT3 mRNA in the eutherian brain. It may be inferred, therefore, albeit tentatively, that at least one MT transcript of *Monodelphis* expresses an isoform with possible functional correspondence to eutherian MT3 in this region.

Opossum MT mRNA has been localised to regions of the tongue epithelium corresponding to sites of mouse MT mRNA expression. However, excessive signal and the scatter attendant upon the use of ^{32}P labelling for probes has confounded unambiguous specification of the cell types involved. Consequently, specific comparison cannot be made with mouse MT1 or MT4 expression in these regions. The cell-specific expression of MT3 in the eutherian tongue has yet to be determined. Iteration of *in situ* hybridisation experiments on the opossum tongue using isotopes ^{35}S or ^{33}P to reduce scatter, as well as transcript-specific probes, will allow fuller comparison of transcription of eutherian and marsupial metallothioneins in the tongue. The extremely high levels of MT transcription in the mammalian tongue have not been previously demonstrated. Further investigation of the phenomenon in other species is warranted, to establish both its prevalence and functional significance. The tongue is proving a very interesting site of metallothionein expression. It is one of very few tissues known to express the complete metallothionein locus in the rat, and is also a principal site for the expression of MT4, which may or may not be the ancestral metallothionein (discussed below). A more comprehensive investigation of the tongue may cast light upon the evolution of MT function.

The expression of MT mRNA in the opossum brain throughout development is comparable to the expression of MT1, MT2 and MT3 in the

eutherian brain. The developmental profile for hepatic MT mRNA in the opossum, however, differs markedly from that of the eutherian, reflecting the very different physiological and metabolic circumstances of the neonates. Further investigation of this comparison should provide useful insight into the role of metallothionein in the developing liver.

It is important to note that the expression of the various opossum metallothioneins has been investigated here only at the level of transcription. No MT protein has yet been demonstrated in the opossum.

CHAPTER 5: PHYLOGENETIC ANALYSIS of the MAMMALIAN METALLOTHIONEINS

5.1 Introduction

The metallothionein genes of the opossum occur as a complex multigene family. This is consistent with what is known of the metallothionein genes of other mammalian species, which also occur as multiple functional isoforms. Such multigene families are believed to evolve by the process of duplication and divergence of an ancestral single-copy gene (Li, 1983). The methods of molecular systematics, which trace the histories of genes and proteins by comparing their sequences, can be applied to decipher this process and to establish the evolutionary relationship of members of gene families within species and between species. In this way, it is sometimes possible to predict the occurrence of a particular isoform within a particular taxon (eg. Hauser *et al*, 1995).

Five opossum cDNAs have been isolated from cDNA libraries of liver and brain. Sequences homologous to *MT3* and *MT4* mRNA have not been detected in opossum tissues nor amplified by PCR. However, negative results are necessarily inconclusive. The occurrence of MT3 in several mammalian species suggests that either this isoform does occur in the closely related marsupial subclass or it is a recently evolved member of the gene family, arising within the ancestor of the eutherian mammals. The *MT4* sequence has been detected in both mouse and human genomes, which implies its occurrence throughout the eutherian mammals. It too may or may not exist in the marsupial lineage. Therefore, the evolution of the metallothionein genes and especially of the mammalian gene family was explored using a molecular systematics approach in the hope that some light might be shed on the existence or otherwise of a marsupial homologue of the eutherian MT3 and MT4 isoforms.

A phylogenetic tree was presented by Prof. J. Kagi in Metallothionein III, (proceedings from the Third International Conference on Metallothionein 1993), which was calculated from the amino acid sequences of 62 vertebrate and invertebrate metallothioneins (Fig. 1.4). This tree implies a closer correspondence between mammalian MT3 and MT4 and the the non-mammalian

vertebrate (birds and fishes) MTs, than between the mammalian MT1s and MT2s and the non-mammalian vertebrate MTs. This would suggest that the MT3 and MT4 isoforms are evolutionarily more ancient than the mammalian MT1 and MT2 isoforms. Prof. Kagi predicts that the latter diverged before the diversification of the mammalian orders. On the basis of this inference, *Monodelphis* could be expected to have both *MT3* and *MT4* genes. Furthermore, it is likely that *MT1* and *MT2* might also coexist in the marsupials, as the split between the marsupials and the eutherian mammals (115 myrs ago - Jancke *et al.*, 1997) predates that of the eutherian radiation (110 myrs ago) by very little time on the evolutionary timescale.

In a recent paper introducing the cDNA sequence of *Ambystoma mexicanum* MT, Saint-Jacques *et al.* (1998) presented a phylogenetic tree calculated for some members of the metallothionein gene family. Unfortunately, no MT3 or MT4 sequence was included so broad generalizations about the overall evolution of the family cannot be drawn. As in the more inclusive tree of Kagi, the mammalian MT1 and MT2 isoforms cluster to the exclusion of the MT proteins of non-mammalian vertebrates. The sequence phylogeny follows the classical vertebrate phylogeny.

The eutherian metallothionein proteins are categorized on the basis of primary structure and tissue distribution into four isoforms. The five opossum metallothioneins derived from the cDNA sequences described in Chapters 3 and 4 have amino acid sequence characteristics, mRNA expression patterns and cognate protein attributes that make it difficult to contain them within the framework of mammalian MT classification as it currently applies. It is difficult to categorize any or all of them as an MT1, MT2, MT3 or MT4 for the reasons given above. The dilemma, therefore, is whether to modify the isoform definitions to accomodate the marsupial and monotreme sequences within an existing group, to introduce further categories into which the marsupial and monotreme metallothioneins can be installed or to re-evaluate the classificatory system altogether. Prof. Kagi, at the Fourth International Metallothionein Meeting in Kansas in 1997, emphasised the need to base any classification of the metallothioneins on "criteria relating to phylogenetics and justifiable differences in protein and/or gene structure" (Kojima *et al.*, 1997). Therefore, it is important

to examine carefully the evolutionary relationships of the vertebrate metallothioneins to ensure a classification system that truly reflects the interrelationships within the family. Furthermore, if we are to evaluate which differences between the proteins are relevant to their classification, the history and distribution of these differences must also be thoroughly described. The amino acid insertion sequences of the MT3 proteins are deemed significant in their classification. It may be possible with phylogenetic analysis to ascertain the evolutionary significance and therefore the classificatory moment, if any, of the insertion sequences in the marsupial and monotreme metallothioneins.

Therefore, the aims of this chapter are:

1. to establish by phylogenetic analysis the evolutionary relationships of the major vertebrate metallothioneins
2. to determine whether the presence of *MT3* and/or *MT4* can be inferred in *Monodelphis*
3. to assess the evolutionary significance of the structure of *Monodelphis* metallothionein sequences

5.1.1 Species phylogeny

Because gene duplication within a multigene family can occur within one organismal lineage and not another, a gene tree is not necessarily congruent with a species tree. However, the history of the gene must be consistent with the species phylogeny. Therefore it is necessary to stipulate the phylogenetic species tree against which the history of metallothionein evolution is to be drawn. The currently accepted phylogeny of the vertebrates is described in Fig 5.1 (Russo, 1997), based on morphological and palaeontological data. This reconstruction corresponds to those derived from sequence comparison of whole mitochondrial genomes (Cummings *et al.*, 1995).

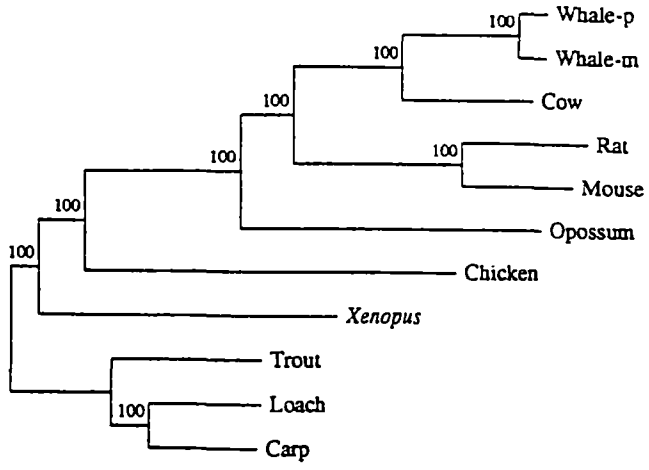


Figure 5.1
A vertebrate phylogeny based on morphological characters and the fossil record (from Russo, 1997). The relative position of the monotremes is discussed in section 1.2 of the Introduction.

As preface to this chapter, a synopsis is given of molecular systematics to assist those readers with little background in phylogenetics.

5.2 Preface - Molecular Systematics

5.2.1 Phylogenetics

Molecular systematics is based on the comparison of homologous characters and the principle that character similarity is indicative of common ancestry, and not of adventitious occurrence. So it is the comparison of homologous characters only that results in meaningful phylogenetic inference. Character likeness arising from causes other than shared ancestry is referred to as "homoplasy", and in the context of molecular sequences is attributible to convergence, reversal or parallelism (Patterson, 1988; Li & Graur, 1991). Homoplasy may confound phylogenetic inference and its effects are therefore minimized as far as possible. The characters of molecular sequences are either constituent nucleotides or constituent amino acid residues, and the homology of

these characters is judged by their position within the context of the molecule - "positional homology". A character of one molecule is the homologue of that in a second molecule if their sites within the molecular sequence are deemed to be the same. Positional homology is established by aligning gene or protein sequences such that regions of high conservation coincide and intervening sequences can be adjusted with maximum correspondence. Alignment is therefore of critical importance in the process of phylogenetic analysis in defining the units of comparison. It is often cited as the most difficult and least understood part of the analysis (Swofford *et al.*, 1996) and has been shown to have a more profound effect on the outcome of an analysis than the choice of analytical method applied to the data (Morrison & Ellis, 1997). The alignment of the class I metallothioneins is relatively unambiguous due to the invariably conserved cysteine residues spread evenly throughout the protein.

A fundamental assumption is that the molecules being compared are truly homologous. In the case of the class I metallothioneins, the extreme sequence conservation establishes unequivocally that the molecules are related. Because metallothioneins occur in gene families within several vertebrate taxa, it is unclear which of the relationships between MT molecules are orthologous (related by direct descent) and which are paralogous (related indirectly through one or more duplication events) (Fitch, 1970). Whereas such clarity is necessary for the evaluation of a species phylogeny, systematic analysis of a gene doesn't require knowledge of these distinctions *a priori*, but may clarify them in its resolution.

5.2.2 Methods of Phylogenetic Inference

A summary of phylogenetic methods and their application is given in the appendix to Chapter 5 (page 143).

Methods of phylogenetic inference fall broadly into two types. The first group, consisting principally of distance matrix methods, applies an algorithm to sequence data and a tree is produced. In the second group of methods, (eg. parsimony, maximum likelihood) an "optimality criterion" is first defined by which a tree can be evaluated, and this criterion can be formally defined by an objective function (Swofford *et al.*, 1996). An algorithm is then used to compute

the value of the optimality criterion for the trees generated from the data. The tree with the best optimality criterion is deemed closest to the "true" tree. The advantage of the second type of method is that the optimality criterion can be chosen to represent a justified evolutionary assumption, and this is de-coupled from the mathematics of its derivation and evaluation (Felsenstein, 1982a). In the distance methods, however, it is the algorithm that defines the tree selection criterion and the meaning of the tree in terms of evolutionary process is obscure.

The relative merits of the methods of inferring phylogenies depend on the degree to which they are sensitive to infringment of the assumptions upon which they are based and the degree to which these assumptions are in accord with real world data (Huelsenbeck & Hillis, 1993). These give rise to systematic error that is discussed for each method separately, in the appendix to Chapter 5, section A5.1.

Maximum parsimony (MP) and maximum likelihood (ML) analyses share the advantage that both are based on criteria with defined evolutionary meaning, whereas distance matrix methods are mathematically based and the phylogenies they produce are arguably more consistent with mathematical processes than evolutionary ones. However, distance matrix methods deal with very large data sets easily, often produce the "true" tree (Olsen, 1987) and if all the assumptions of the distance corrections are met, will be consistent (Huelsenbeck & Hillis, 1993). Parsimony analysis is subject to inconsistency, which is the tendency of an estimate to converge on the wrong conclusion as more data are added (Felsenstein, 1978). Inconsistency occurs in situations where data have not been corrected for multiple or superimposed substitutions at sites (Penny *et al.,* 1996), giving rise to the phenomenon of "long branch attraction" (discussed below, in section A5.1.2). ML is not subject to problems of inconsistency (Kuhner & Felsenstein, 1994; Huelsenbeck, 1995a,b). More pragmatic reasons for choosing one method over another often involve computation constraint: some methods (parsimony branch-and-bound, ML) are so computationally expensive that only a limited number of sequences can be addressed in a single analysis. Other methods (parsimonious heuristic searches) sacrifice the assured discovery of all optimal trees for fast computation and an ability to handle large numbers of taxa.

It has been suggested those branching patterns consistently found by different methods are likely to be the most reliable (Miyamoto & Cracraft, 1991; Hillis & Huelsenbeck, 1994) and that congruence between phylogenies resulting from different methods of tree-construction supports the credibility of those phylogenies (Kim, 1993; Flook & Rowell, 1997). On this basis, distance, MP and ML methods were used to evaluate the relationships between the metallothionein genes, and the resultant phylogenies were assessed and compared.

It is recommended that any reader unfamiliar with the techniques of phylogenetic analysis read the appendix to Chapter 5 before continuing onto the following sections. These sections are cross-referenced to the relevant sections of the appendix to facilitate greater clarity. A list of definitions of phylogenetic terms used here is also provided at the end of the appendix to Chapter 5 (A5.11).

5.3 RESULTS

5.3.1 Choice of Data Sets and Computational Methods

Phylogenetic analyses require choices to be made between differing computational methods, and their application to specific subsets of the available data. Each set of choices relies on particular assumptions, the formulations of which must be consistent with the data and the context of the analysis. In this section, these various choices and assumptions are outlined and justified.

5.3.1.1 Sequences Used in Analyses

DNA sequences were taken from the Genbank and protein sequences from Kagi, 1993 (and references therein), unless otherwise specfied. Species names, accession numbers and sources for DNA and protein sequences are given in Tables 5.1 and 5.2, respectively. DNA sequences and derived amino acid sequences for opossum and echidna metallothioneins are taken from Chapter 3.

MT sequences were aligned using conserved cysteines as reference points. The alignment of vertebrate metallothionein nucleotide sequences is given in Appendix I, and the alignment of amino acid sequences used is given in Appendix II. The data sets described in Apendices I and II are hereunder referred

to as the "complete" data sets and constitute the majority of those vertebrate metallothionein sequences available to the author at the time of analysis (with the exception of some fish sequences). Subsets of these taxa are defined for different analyses below. Data sets analysed here are first evaluated for non-random structure using the index of character covariance, the g_1 statistic (as described in section A5.2), derived from 1,000 random trees, using PAUP 3.1 (Swofford, 1993). The value of g_1 is recorded with each reconstruction (Figs. 5.2-5.16). It can be seen in Figs. 5.2-5.16 that all data sets analysed here conform to the requirement for non-random structure, defined in Table 5.2 (section A5.2).

Although the number of bootstrap replicates required for a statistically accurate result (1% at P=95) is stipulated at 2,000 (Hedges, 1992), a preliminary analysis of 100 replicates was performed in each case below, on the basis that computation time is greatly reduced and a fairly accurate idea of confidence can be established for branches with the fewer replicates (Felsenstein, 1985; Hedges, 1992; Efron *et al*, 1996). The use of "bootstrapping" to estimate the confidence level of a particular phylogenetic reconstruction is discussed in section A5.4, and the concept of the bootstrap proportion, P, described.

5.3.1.2 Data Subsets

Both the branch-and-bound algorithm of parsimony and the algorithms of ML analysis are constrained by computing requirements to a maximum number of sequences, especially when bootstrap confirmation is required. Furthermore, parsimony is sensitive to the spectre of inconsistency if character and taxon sampling is indiscriminate. (Inconsistency is discussed in section A5.1.2, and the effects of taxon sampling in section A5.7). For these reasons, subsets of the complete data set were assembled to conform to the stringencies of the programs and the required outcomes. The choice of representative taxa for analysis is discussed in section A5.7. Preliminary heuristic searches were performed using PAUPSearch in the interactive mode through ANGIS (Australian National Genomic Information Service - a data base manipulation facility sited at the University of Sydney, Australia) on both the amino acid and nucleic acid complete data sets (results not shown). Calculated phylogenies confirmed certain

invariant relationships also demonstrated in the distance matrix trees described in section 5.4.1:

* human MT4 and mouse MT4 are monophyletic
* the eutherian MT3s are monophyletic
* the opossum MTs are monophyletic
* the echidna MTs are monophyletic
* each individual isoform within the rodents (rat, mouse and hamster) is monophyletic

These invariably monophyletic groups can therefore be represented by a single taxon member where there are constraints upon the number of taxa that can be processed by an algorithm. For instance, only one representative rodent sequence is ever used for each isoform.

5.3.1.2.1. non-redundant data set (nr)

As has been suggested (Milinkovitch *et al.*, 1996), the effects of homoplasy on a tree can be reduced if variation within the taxa represented is adequately sampled (see section A5.7.3 for a discussion of the effects of redundant taxa). From the distance matrix TREE 1 (discussed in full below in section 5.3.2.1), it can be seen that the genetic distances that separate the vertebrate metallothioneins vary greatly. Distances, and hence variation, between two fish metallothioneins can be much greater than between two mammalian metallothioneins. Therefore, a non-redundant data set was constructed (as described in appendix A5.7.3) by removing those sequences which fall within close proximity to another sequence, when both belong to the same taxonomic unit or isoform group. The non-redundant set of taxa is illustrated in Fig. 5.2, with the corresponding g_1 statistic. This data set was later expanded to include other isoforms, such as human MT2 and MT1a, to test species sensitivity (refer to appendix A5.7.2). The two protein isoforms found in pigeon were both included in the protein analysis together with the chicken sequence, because the co-occurrence of two MT proteins is unusual within the birds (Lin *et al.*, 1990a), and their relationship is therefore of interest.

The outgroup taxa were specified initially as the fish metallothioneins, on the presumption of antiquity, monophyly and in accord with the species

phylogeny given in Fig. 5.1 (Russo, 1997). The amphibian taxa *Xenopus* and *Ambystoma* were subsequently added to the outgroup to compare the effects on ingroup relationships. All combinations of fish species as outgroup gave rise to identical ingroup relationships under the same conditions. The significance of outgroup choice is discussed in appendix A5.6.

5.3.1.2.2 non-redundant data set, expanded (nrx)

In this subset, a maximum of twenty metallothionein sequences were chosen. Because the relationships between the various mammalian isoforms are of primary interest, at least two representatives of eutherian MT1 and MT2 are included. Both human and mouse MT3 and MT4 sequences are included to ensure true representation of the group as far as possible and to avoid any effects of species sensitivity. Limiting the number of eutherian sequences can be justified on several grounds:

1. there is a disproportionate number of eutherian sequences available - tempting the researcher to use more than necessary;
2. imbalance in branch lengths is possible with the threat of long branch attraction (described in section A5.1.2);
3. there is presumed to be considerable gene conversion between members of the MT multigene families of the eutherian mammals (Schmidt *et al.*, 1985; Hamer, 1986; Peterson *et al.*, 1988)), which could confuse phylogenetic signal.

The mouse, with its simple gene family, is an obvious choice to derive details of gene family evolution and the mouse isoforms are usually included. To avoid problems of species-sensitivity, human and/or another eutherian sequences are also included.

A single sequence from each of the marsupial and monotreme species is included as each group is demonstrably monophyletic and the genetic distance between the isoforms small. The two available amphibian sequences and the four fish species calculated to be non-redundant in terms of corrected genetic distances (see section A5.7.3) in the context of the complete datatset are also included (pike, loach, cod and perch). The chicken MT sequence represents the order Aves in the analysis and the duck, turkey, quail and muscovy sequences

omitted due to their remarkable near-identity with the chicken sequence (Lin & Huang, 1990). Both pigeon isoforms are included in the protein analyses for the reasons given (above) for the non-redundant data set. From two to four more sequences were added into these computations to test for species bias. Outgroups were specified in the same way as for the non-redundant data set and all combinations of outgroups.

5.3.1.3 Distance Analysis

Distance analysis is reviewed in section A5.1.1 of the appendix to Chapter 5. Because computation time is not limiting in distance matrix methods of phylogenetic inference and the algorithms are not sensitive to number of, or variability within, taxa, distance matrix trees were established for the complete data sets, described in Appendices I and II. Duck and muscovy sequences have been omitted in the calculation of amino acid-based trees, as they are identical to that of chicken MT.

The PHYLIP package version 3.5 (Felsenstein, 1995) in the interactive format was used through WebANGIS (the ANGIS facility accessed through the internet) to compute genetic distance matrices and derive phylogenetic trees based on these. Bootstrapping of data sets was done with the program **eseqboot**, with 100 replications. The multiple data sets so generated were then processed through the **ednadist** or **eprotdist** programs, for DNA and protein data sets, respectively. The neighbour-joining algorithm **eneighbor** was used to construct unrooted trees for all matrices. Trees were rooted *a posteriori* with the fish species, pike. The resultant file of trees was reduced to a majority rule consensus tree in each case by the program **econsense**. The concept of the consensus tree is discussed in section A5.3. Bootstrap support for each branch is indicated numerically as the percentage of replications that support the group descendent from that branch.

For DNA data sets, the program **ednadist** produced distance matrices under three different models of nucleotide substitution: the Kimura 2 parameter model (K-2-P), with and without the Jin & Nei (JN) option, and the Jukes-Cantor (JC) model.

The LogDet transformation was accessed through the PAUPSEARCH package available in the interactive format through WebANGIS, and applied to the complete DNA data set. The neighbour-joining algorithm, **eneighbor**, was used to compute the tree from the LogDet distance matrix.

5.3.1.4 Parsimony Analysis

Parsimony analysis and its attributes are reviewed in section A5.1.2 of the appendix to Chapter 5.

Parsimony analyses on the DNA extended non-redundant (DNAnrx) data set were performed using the programs of the PHYLIP package through WebANGIS. Unweighted parsimony using the branch and bound algorithm **ednapars** on informative characters only in 100 bootstrap replicates (**eseqboot**) produced a modified 50% consensus tree, using the **econsense** program. Aspects of character weighting in phylogenetic analysis are discussed in section A5.5. It is a property of the program to recognize a deletion of N bases in length as N separate deletion events (Felsenstein, 1995). To overcome this, gaps in the sequence alignment (corresponding to insertions and deletions) were treated as missing data and removed from the computation. The DNAnrx data set was then modified by the effective removal of the third position of each codon by down-weighting to zero. Trees were rooted *a posteriori* with the fish species. The differentially weighted DNAnrx data set was then analysed in the same manner.

The program **eprotpars** was used to analyse 100 bootstrap replicates of the extended non-redundant amino acid data set, and a modified 50% consensus tree calculated.

5.3.1.5 Maximum Likelihood Analysis

The maximum likelihood method of phylogenetic analysis is described in section A5.1.3 of the appendix to Chapter 5. Using the program **ednaml** in the interactive mode of PHYLIP version 3.5 through WebANGIS, and 100 data sets generated from the DNAnr and DNAnrx data sets by bootstrapping with the program **eseqboot**, maximum likelihood trees were generated with a ts:tv weighting of 2.0 (ts:tv weighting is discussed in appendix A5.5.2). The trees

were rooted on the fish species. The 100 resultant trees were reduced to a consensus using the program **econsense.**

The DNAnrx data set and larger data sets were analysed with the program DNAML 3.5 in PHYLIP through the interactive mode of ANGIS, to access the option to specify rate heterogeneity across character sites through varying multiple rate categories (as described in section A5.1.3).

The PROTML program of PHYLIP version 3.5 (Adachi & Hasegawa, 1992) was used through ANGIS in the interactive mode, to calculate a phylogeny from the modified non-redundant protein data set. The bootstrap option was not invoked to minimize the already protracted computation time.

5.3.2 Phylogenetic Reconstructions

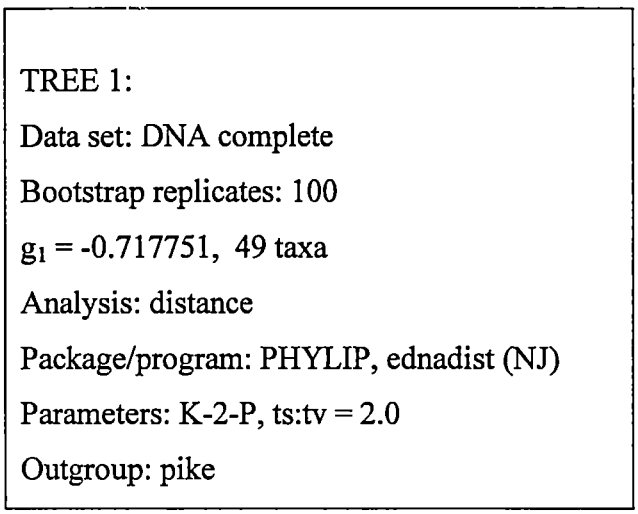
In this sections, the trees calculated by phylogenetic analysis are presented and discussed.

5.3.2.1 Distance Matrix

Distance-based phylogenies were calculated from the complete DNA data set using JC, K-2-P and JN transformations (see section A5.1.1) and a transversion:transition (ts:tv) weighting of 2.0 (A5.5.2). The significance of the transversion: transition ratio in character weighting is reviewed in section A5.5.2. (The JC transformation has, by definition, a ts:tv=1.0). These trees conform with one another and are presented as TREE 1 (Fig. 5.2). Reading phylogenetic trees is described in section A5.10 of the appendix to Chapter 5. *MT1* and *MT2* form a monophyletic group or "clade". *MT3* sequences appear to be ancestral to all other mammalian sequences, including the marsupial and monotreme metallothioneins. Eutherian *MT4*s form a sister group to the eutherian *MT1* + *MT2* clade. Bootstrap values vary. A relatively high value of 73% supports the monophyly of the *MT1* and *MT2* monophyly. However, very low values support most of the clades.

When ts:tv = 20.0, which presumes a high level of saturation in transitional changes consistent with large genetic distances between molecules, the phylogenies (TREE 2 - Fig. 5.3) derived from the different transformations (K-2-P, JN) differ from TREE 1. The *MT4* sequence is ancestral to all other

Figure 5.2



Distance matrices were derived from the complete DNA data set with 100 bootstrap replicates, under Kimura-2-parameter transformation with ts:tv=2.0. When the JC and JN transformations were used, identical trees were produced. Trees were constructed with the neighbor-joining algorithm, with a randomized order of input and reduced to a modified 50% majority rule consensus tree, rooted *a posteriori* on the fish species, pike. Numbers at nodes (points of bifurcation) represent the bootstrap proportion (P) supporting that clade or bifurcation, in this and following trees.

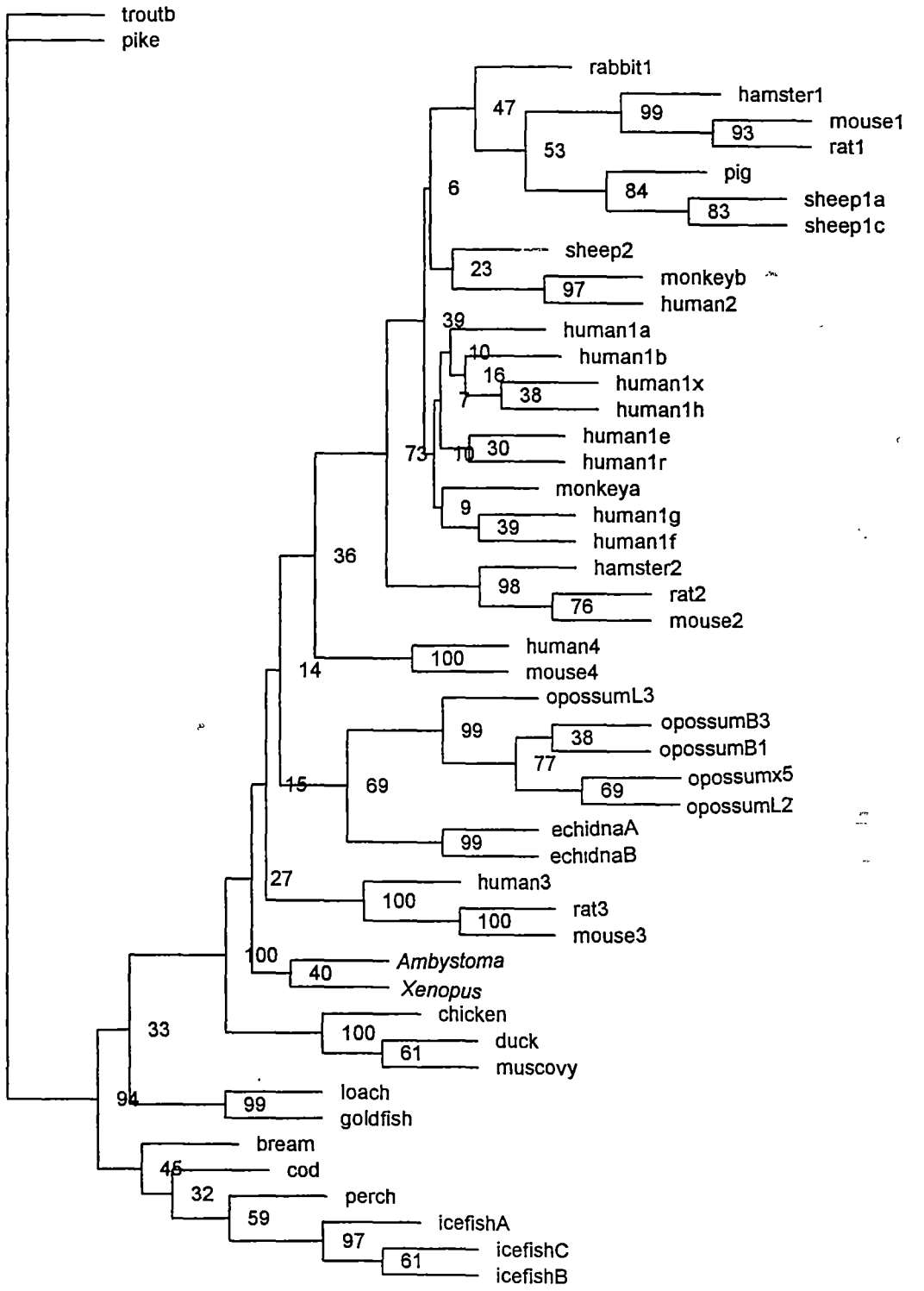
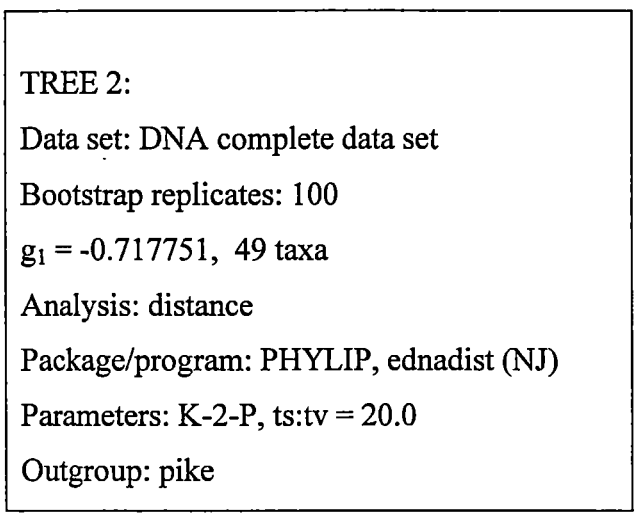
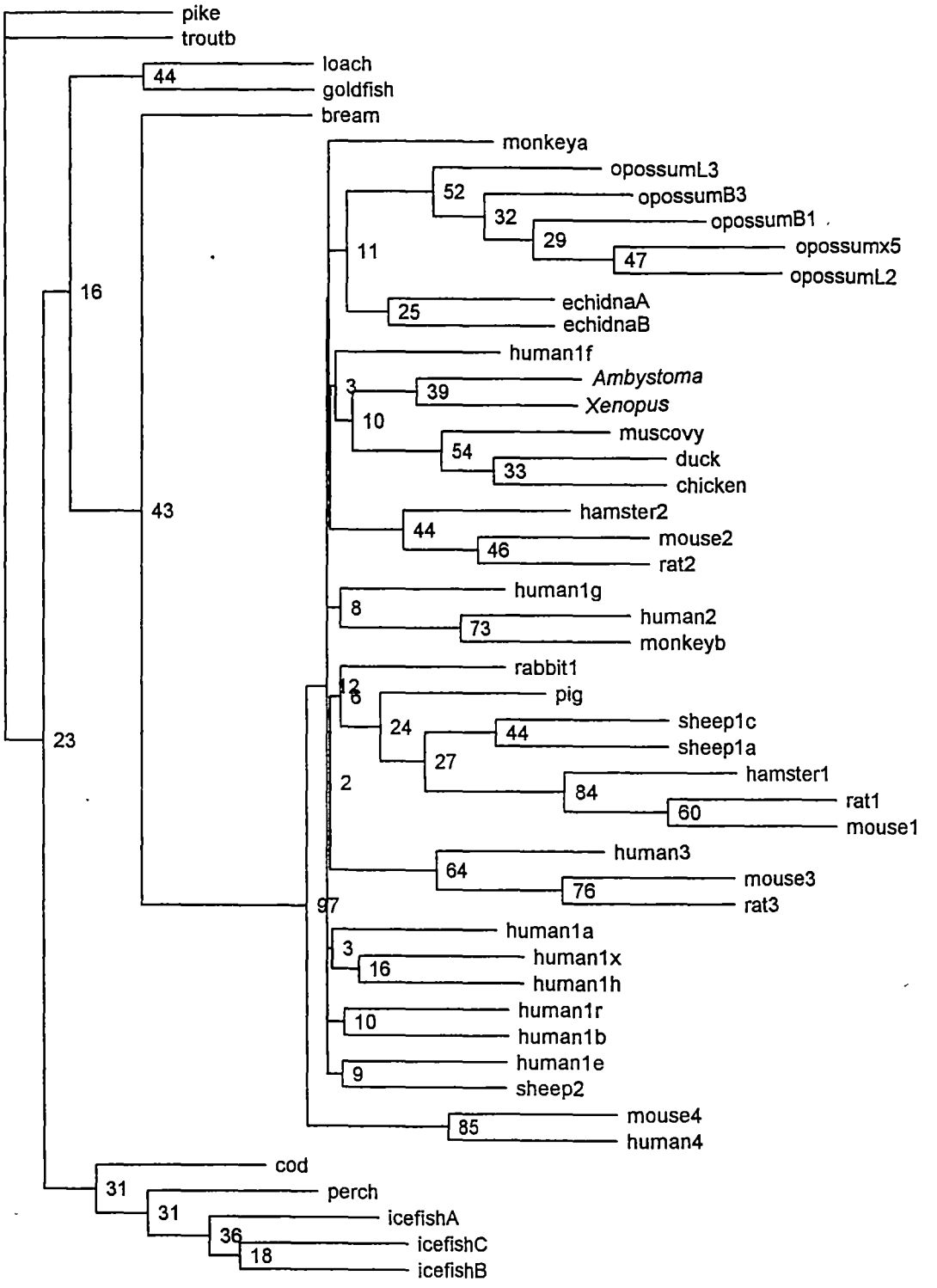

troutb
pike
rabbit1
hamster1
mouse1
rat1
pig
sheep1a
sheep1c
sheep2
monkeyb
human2
human1a
human1b
human1x
human1h
human1e
human1r
monkeya
human1g
human1f
hamster2
rat2
mouse2
human4
mouse4
opossumL3
opossumB3
opossumB1
opossumx5
opossumL2
echidnaA
echidnaB
human3
rat3
mouse3
Ambystoma
Xenopus
chicken
duck
muscovy
loach
goldfish
bream
cod
perch
icefishA
icefishC
icefishB
47
99
93
53
84
83
6
23
97
39
10
16
7
38
73
10
30
9
39
36
98
76
100
14
99
38
77
69
15
69
99
27
100
100
100
40
33
100
61
94
99
46
32
59
97
61

Figure 5.3



Tree 2 was derived using the same parameters as for tree 1, using the K-2-P transformation, except ts:tv=20.0.


pike
troutb
loach
goldfish
bream
monkeya
opossumL3
opossumB3
opossumB1
opossumx5
opossumL2
echidnaA
echidnaB
human1f
Ambystoma
Xenopus
muscovy
duck
chicken
hamster2
mouse2
rat2
human1g
human2
monkeyb
rabbit1
pig
sheep1c
sheep1a
hamster1
rat1
mouse1
human3
mouse3
rat3
human1a
human1x
human1h
human1r
human1b
human1e
sheep2
mouse4
human4
cod
perch
icefishA
icefishC
icefishB
44
52
32
29
47
11
25
16
3
39
10
54
33
43
44
46
8
73
12
6
24
44
27
2
84
60
64
76
97
3
16
10
9
85
31
31
36
18
23

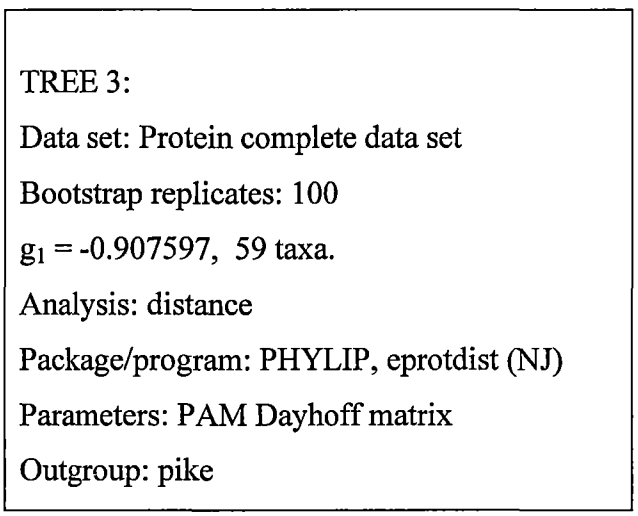
mammalian *MT*s. However, the placement of bird and amphibian sequences among the eutherian *MT1* and *MT2* sequences by these two transformations is untenable in terms of the species phylogeny of Fig. 5.1. This suggests that a ts:tv of 20 defines a degree of transitional saturation that is not supported by the data.

When distance analysis is applied to protein sequences (TREE 3 - Fig. 5.4), the eutherian MT3 and MT4 isoforms cluster with the non-eutherian protein sequences from birds, marsupials and monotremes. The eutherian MTs 1 and 2 form a monophyletic group. (The placement of the rodent MT1 group is inconsistent with the species phylogeny of Fig. 5.1, and other phylogenetic reconstructions presented here, suggesting it is an artefact of the analysis.) It is noteworthy that the pigeon MT1 isoform clusters with the MT4 proteins with bootstrap support of 51%, in this context of otherwise very low bootstrap values, and that the pigeon MT2 isoform clusters with the chicken MT at P=100.

When the categories transformation for amino acids is used (Felsenstein 1995) with a ts:tv weighting = 2.0 to produce TREE 4a (Fig. 5.5), MT4 is clearly the ancestral mammalian metallothionein, clustering with the pigeon MT1 isoform (P= 30). The marsupial and monotreme MTs cluster with the eutherian MT3s, to the exclusion of all MTs1 and 2. When ts:tv = 20.0, MT4 is no longer clearly the most ancient of the mammalian MTs (TREE 4b - Fig. 5.6). However, the MT4 and MT3 isoforms cluster with the marsupial and monotreme MTs, and those of the birds, to the exclusion of the eutherian MTs1 and 2, and this dichotomy is supported with a bootstrap value of 99%. Increasing the ts:tv weighting diminishes the contribution to the phylogenetic signal of the transitional changes in the sequence and resolution will be lost first at sequences still close enough in time to be unsaturated at some sites. The assumption of ts:tv = 20.0 causes distortion in the nucleic acid distance matrix analysis (Tree 2), so recourse to the lower ts:tv would seem prudent. Overall, the protein trees support the separation of the eutherian MTs1 and 2 from the cluster of MT3, MT4, marsupial, monotreme and bird MTs.

The position of eutherian *MT4* in the DNA-based reconstruction of TREE1 contradicts the placement of MT4 as the ancestral eutherian metallothionein in the protein-based trees (TREES 3-4). Base compositional bias is known to engender artefacts in phylogenetic reconstructions, as discussed in

Figure 5.4



Distance matrices were derived from the complete amino acid data set, after 100 bootstrap replications, using the PAM Dayhoff substitution matrix. Trees were constructed with the neighbor-joining algorithm with randomised order of input and reduced to a modified 50% consensus tree, rooted *a posteriori* on the fish species, pike.

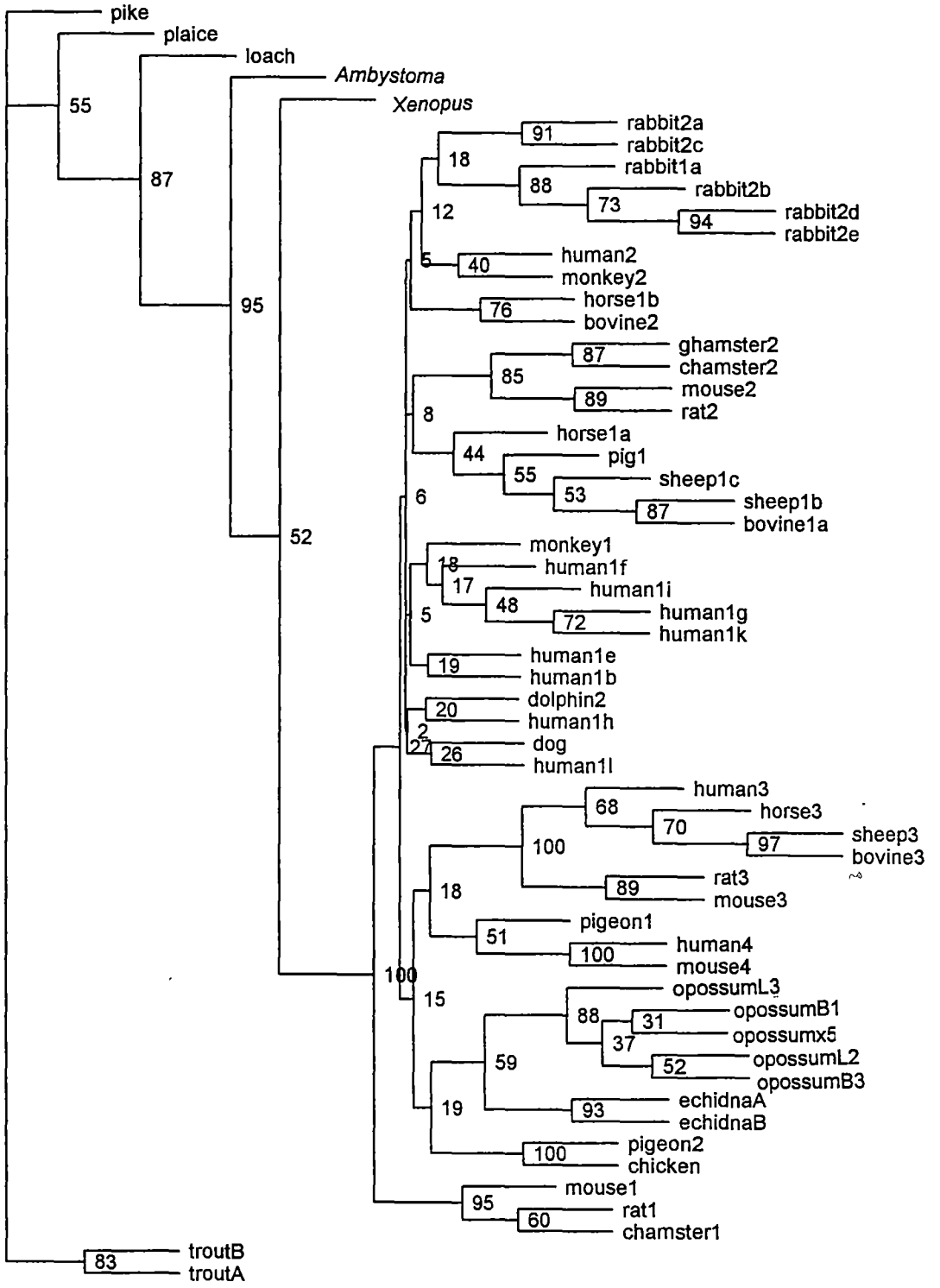
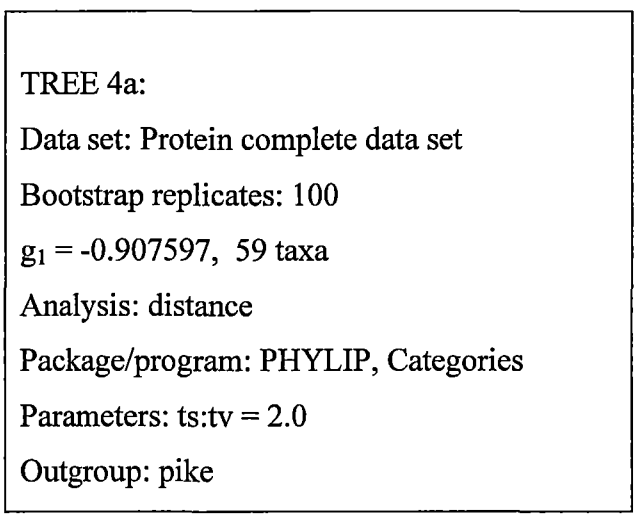

pike
plaice
loach
Ambystoma
Xenopus
55
87
95
52
91
rabbit2a
rabbit2c
18
rabbit1a
88
rabbit2b
73
94
rabbit2d
rabbit2e
12
5
40
human2
monkey2
horse1b
76
bovine2
87
ghamster2
chamster2
85
mouse2
89
rat2
8
horse1a
44
pig1
55
sheep1c
53
87
sheep1b
bovine1a
6
monkey1
18
human1f
17
human1i
48
human1g
72
human1k
5
human1e
19
human1b
dolphin2
20
human1h
2
27
dog
26
human1l
human3
68
horse3
70
97
sheep3
bovine3
100
rat3
89
mouse3
18
pigeon1
51
human4
100
mouse4
100
opossumL3
15
88
opossumB1
31
opossumx5
37
59
opossumL2
52
opossumB3
echidnaA
19
93
echidnaB
pigeon2
100
chicken
mouse1
95
rat1
60
chamster1
troutB
83
troutA

Figure 5.5



The complete amino acid data set was bootstrapped (100 replicates) and distance matrices computed using the "categories" method of Felsenstein (1995) with ts:tv=2.0. Trees were constructed with the neighbor-joining algorithm with randomised order of input and reduced to a modified 50% consensus tree, rooted *a posteriori* on the fish species, pike.

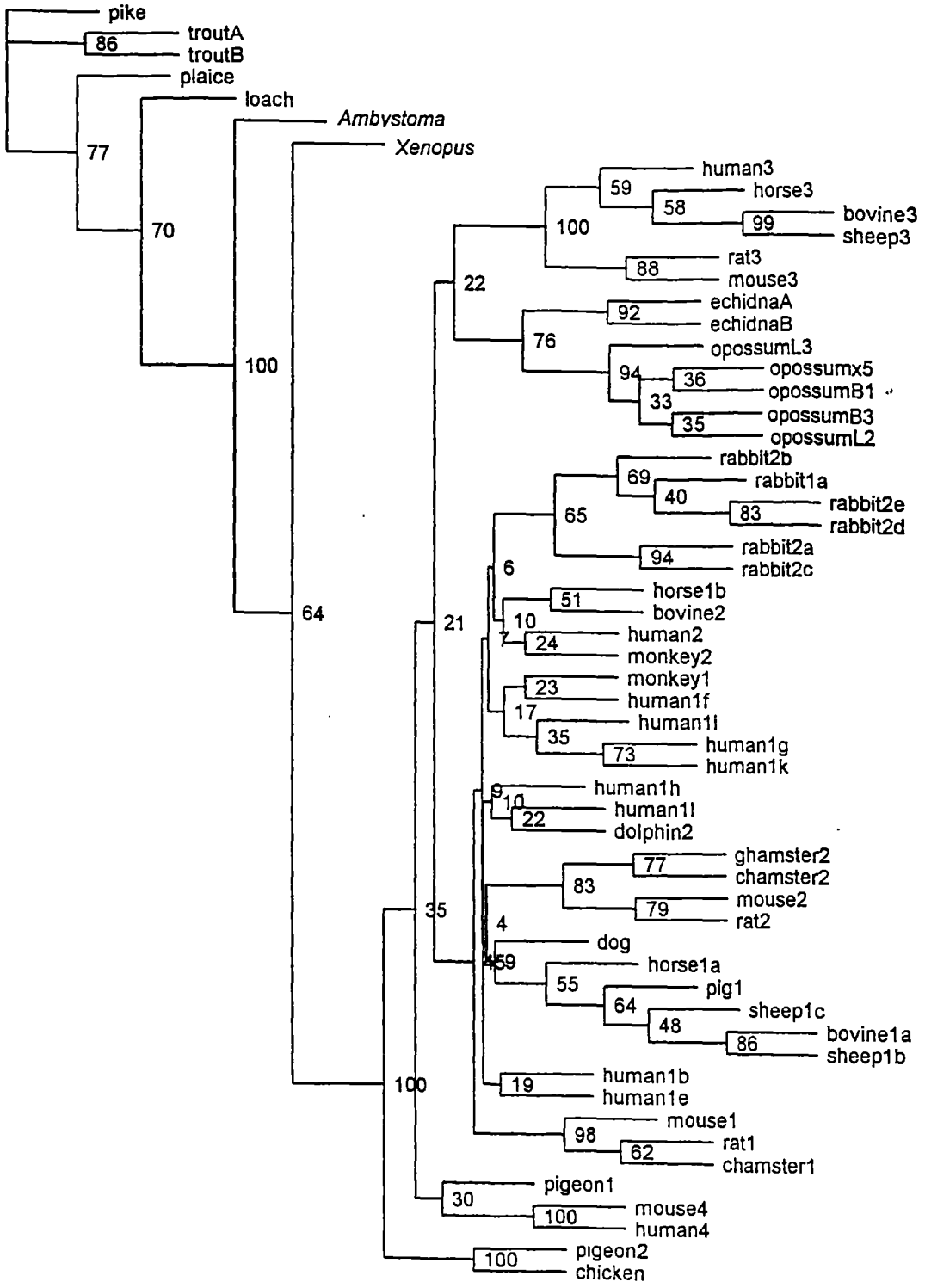
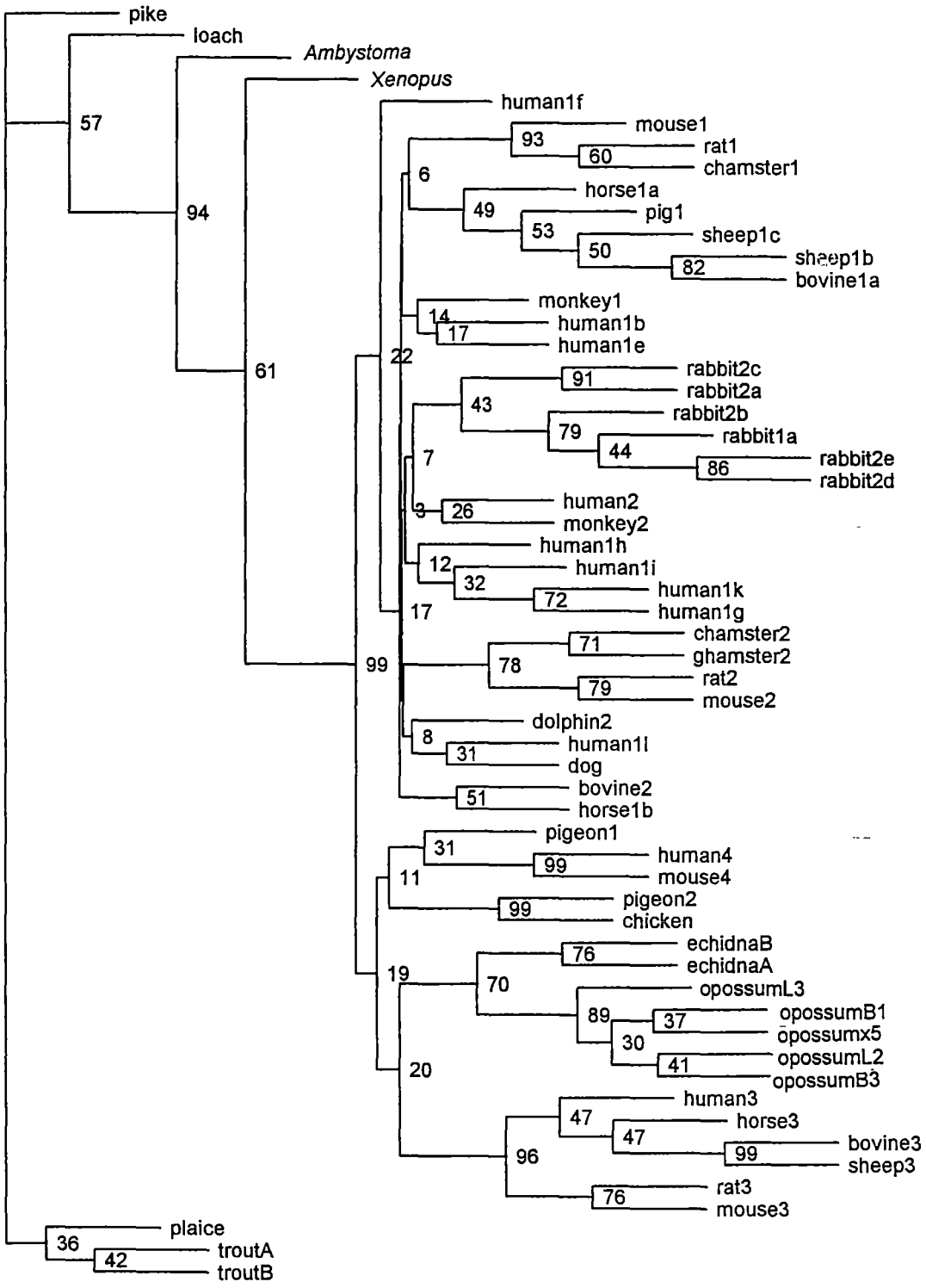

pike
troutA
86
troutB
plaice
loach
Ambystoma
Xenopus
77
70
100
64
human3
59
horse3
58
bovine3
99
sheep3
100
rat3
88
mouse3
22
echidnaA
92
echidnaB
76
opossumL3
94
opossumx5
36
opossumB1
33
opossumB3
35
opossumL2
rabbit2b
69
rabbit1a
40
rabbit2e
83
rabbit2d
65
rabbit2a
94
rabbit2c
6
horse1b
51
bovine2
10
human2
7
24
monkey2
monkey1
23
human1f
17
human1i
35
human1g
73
human1k
9
human1h
10
human1l
22
dolphin2
ghamster2
77
chamster2
83
mouse2
79
rat2
35
4
dog
59
horse1a
55
pig1
64
sheep1c
48
bovine1a
86
sheep1b
100
human1b
19
human1e
mouse1
98
rat1
62
chamster1
pigeon1
30
mouse4
100
human4
pigeon2
100
chicken

Figure 5.6

TREE 4b:
Data set: Protein complete data set
Bootstrap replicates: 100
g_1 = -0.907597, 59 taxa
Analysis: distance
Package/program: PHYLIP, Categories
Parameters: ts:tv = 20.0
Outgroup: pike

The complete amino acid data set was analysed as for TREE 4a, except with ts:tv=20.0.


pike
loach
Ambystoma
Xenopus
57
94
61
human1f
mouse1
93
rat1
60
chamster1
6
horse1a
49
pig1
53
sheep1c
50
sheep1b
82
bovine1a
monkey1
14
human1b
17
human1e
22
rabbit2c
91
rabbit2a
43
rabbit2b
79
rabbit1a
44
rabbit2e
7
86
rabbit2d
3
26
human2
monkey2
human1h
12
human1i
32
human1k
72
human1g
17
chamster2
71
ghamster2
99
78
rat2
79
mouse2
dolphin2
8
human1l
31
dog
bovine2
51
horse1b
pigeon1
31
human4
99
mouse4
11
pigeon2
99
chicken
echidnaB
76
echidnaA
19
70
opossumL3
89
opossumB1
37
opossumx5
30
opossumL2
41
20
opossumB3
human3
47
horse3
47
bovine3
99
96
sheep3
rat3
76
mouse3
plaice
36
troutA
42
troutB

section A5.8.1 of the appendix to Chapter 5. To assess any distortion introduced by base compositional bias in the complete DNA data set, the LogDet transformation (A5.1.1) was applied to calculate TREE 5 (Fig. 5.7). In this reconstruction, *MT4* is the gene ancestral to all other mammalian metallothioneins. The *MT3* sequences form a monophyletic group arising from the *MT4* sequences, as do the opossum and echidna sequences. The *MT1* and *MT2* sequences of the eutherian mammals also constitute a monophyly. This reconstruction conforms with the protein-based most-parsimonious trees in the ancestral position of *MT4*. Therefore, the placement of *MT4* in a monophyly with the eutherian *MT1* and *MT2* sequences in TREE1 can be attributed to the effects of base compositional bias.

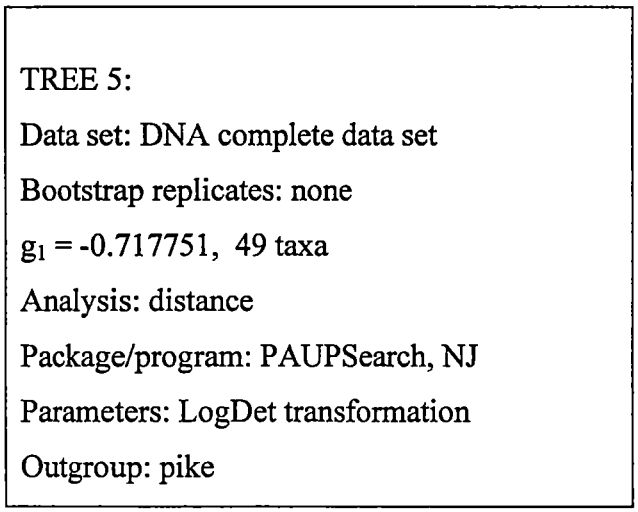
Summary of distance analyses

Some relationships are invariant through all distance matrix reconstructions:

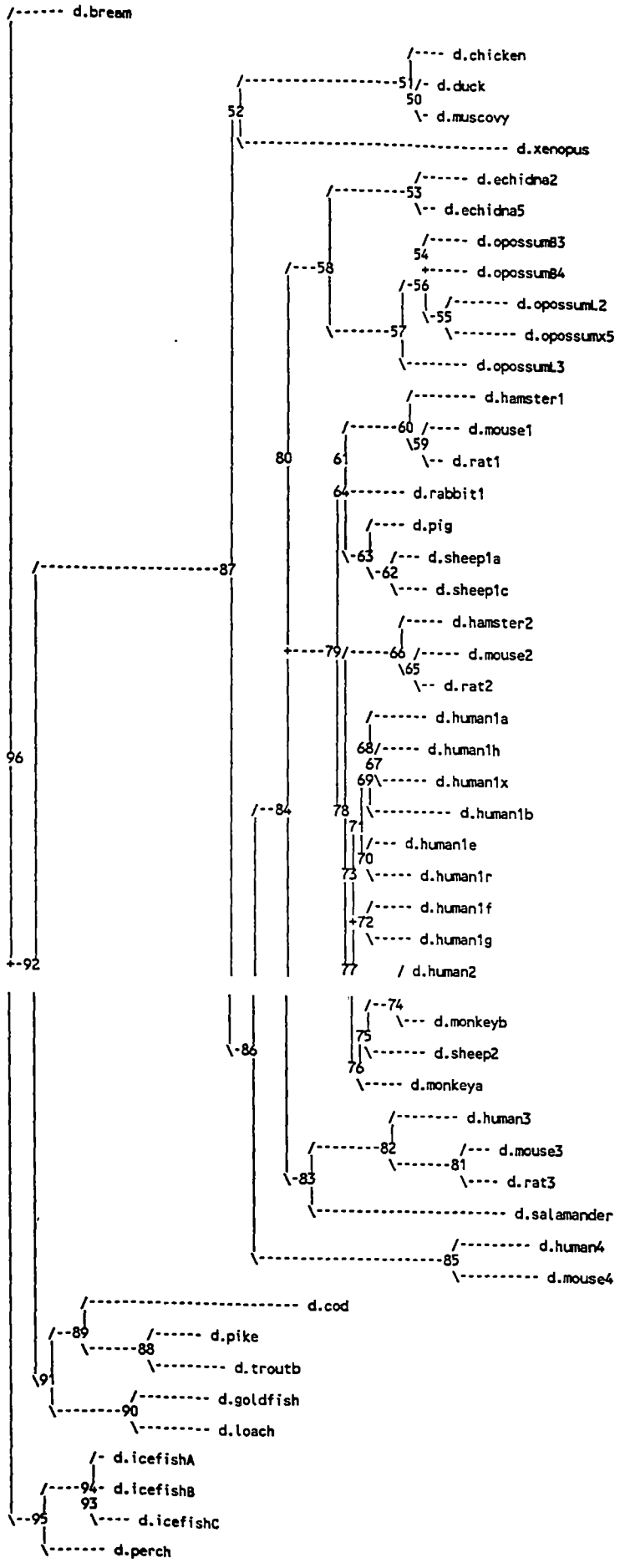
1. MT1s and MT2s together always form a monophyletic group (disregarding the aberrant rodent MT1 sequences of TREE 3)
2. opossum MTs are always monophyletic
3. echidna MTs are always monophyletic
4. opossum and echidna MTs together form an invariant clade
5. MT3s are always monophyletic
6. MT4s are always monophyletic

These data confirm that the MT isoforms are genetically-based entities, maintained through evolution. Any lack of segregation between eutherian MT1 and MT2 sequences on the trees can be attributed to the gene conversion noted for several mammalian *MT* gene families. Uncorrected DNA-based reconstructions place *MT3* as the most ancient of the mammalian *MT*s. However, when the effects of base composition are removed from the DNA-based analyses, *MT4* becomes the ancestral mammalian metallothionein. Protein-based trees cite MT4 as the ancestor of the mammalian isoforms. In both DNA-based and protein-based trees, the marsupial and monotreme genes cluster with the eutherian MT3s. Only for the LogDet transformed data do the *MT3*, *MT1/2* and marsupial/monotreme *MT*s remain unresolved as sister clades.

Figure 5.7



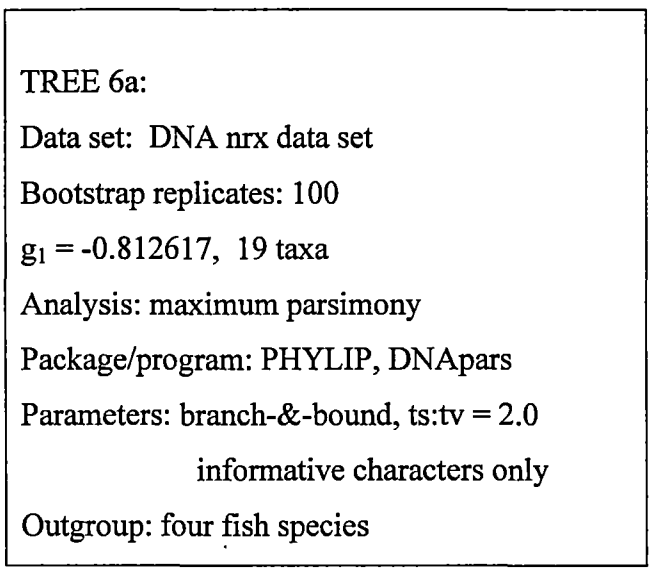
A data matrix was derived from the complete DNA data set using the LogDet transformation, and a tree constructed from the matrix using the neighbor-joining algorithm.


d.bream
d.chicken
d.duck
d.muscovy
d.xenopus
d.echidna2
d.echidna5
d.opossum83
d.opossum84
d.opossumL2
d.opossumx5
d.opossumL3
d.hamster1
d.mouse1
d.rat1
d.rabbit1
d.pig
d.sheep1a
d.sheep1c
d.hamster2
d.mouse2
d.rat2
d.human1a
d.human1h
d.human1x
d.human1b
d.human1e
d.human1r
d.human1f
d.human1g
d.human2
d.monkeyb
d.sheep2
d.monkeya
d.human3
d.mouse3
d.rat3
d.salamander
d.human4
d.mouse4
d.cod
d.pike
d.troutb
d.goldfish
d.loach
d.icefishA
d.icefishB
d.icefishC
d.perch

5.3.2.2 Parsimony

The complete DNA data set is too large to be analysed by parsimony. Therefore, subsets of these data have been used as described in section 5.3.1.2. The unweighted extended, non-redundant DNA data set (DNAnrx) was analysed with the branch-and-bound algorithm of the PHYLIP package (TREE 6a- Fig. 5.8), with the four fish species as outgroup. The eutherian *MT4* sequences cluster with the *MT1/2* sequences with little bootstrap support (P=18). (The concept of bootstrap support, and an explanation of the bootstrap proportion P, is outlined in section A5.4). Opossum and echidna *MT*s cluster with the eutherian *MT*3 sequences, once again with little bootstrap support (P=18). To test for species-sensitive effects on the tree, each taxon and taxon-group was selectively removed and the tree recalculated. When one of the two amphibians (*Xenopus* or *Ambystoma*) is removed, eutherian *MT4* becomes the sequence ancestral to the mammalian *MT*s, with a bootstrap support of 47%. When both amphibian sequences are removed simultaneously, this support reaches 100% (TREE 6b - Fig. 5.9). Removal of the chicken *MT* sequence has a similar effect. In these trees, marsupial and monotreme *MT*s cluster with eutherian *MT3*s. Exclusion of various mammalian sequences, and/or exchange with others from the complete data set, has no obvious effect on the phylogeny. All combinations and permutations of the various fish sequences as outgroup maintained a stable ingrouping, indicating that the fish provide an appropriate outgroup for this reconstruction (A5.6). When the amphibian sequences are added into the outgroup, the mammalian *MT*s maintain their relative positions of TREE 6a, but the chicken *MT* sequence moves to a site ancestral to the amphibian sequences, untenable in terms of the species phylogeny. The position of *MT4* on the DNA parsimony tree, therefore, seems to be sensitive to the presence of the amphibian and bird sequences. Analysis of the G+C content of the *MT* sequences is given in Table 5.1. The amphibian and bird sequences represent the two extremities on the spectrum of values. This may be a factor introducing bias into the analysis (see A5.8.1). Fish *MT* sequences, on the other hand, span a wide range of values which overlap those of most other groups. To overcome the problem of base

Figure 5.8



Tree 6a was constructed using the maximum parsimony optimality criterion and the branch-and-bound algorithm, on the informative characters only of the extended DNA (nrx) data set. Sequence gaps were treated as "missing" data. No weighting was applied to characters or sites. Bootstrap = 100 replicates. A modified 50% majority rule consensus tree was calculated, and the tree rooted *a posteriori* on the four fish sequences.

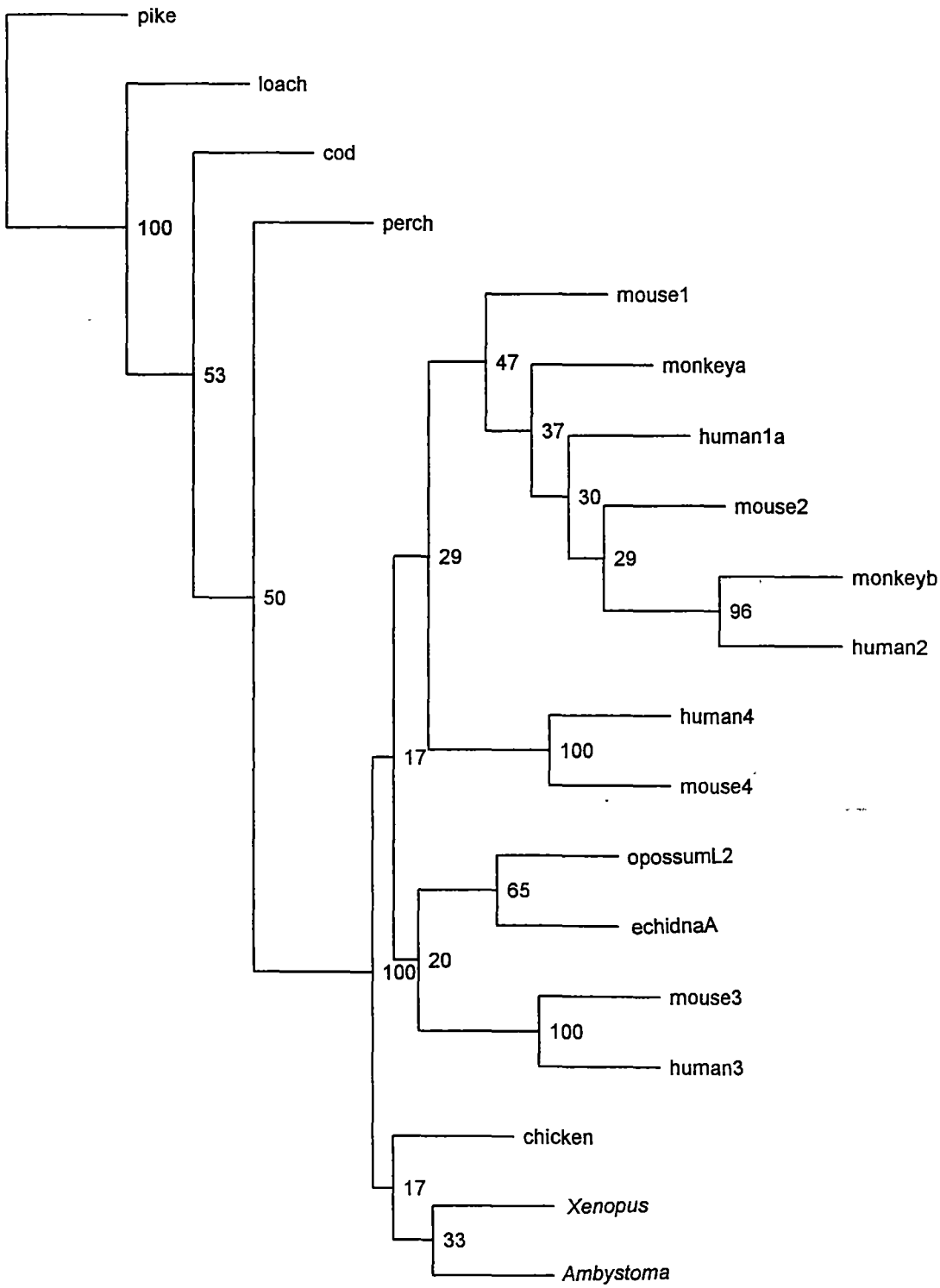
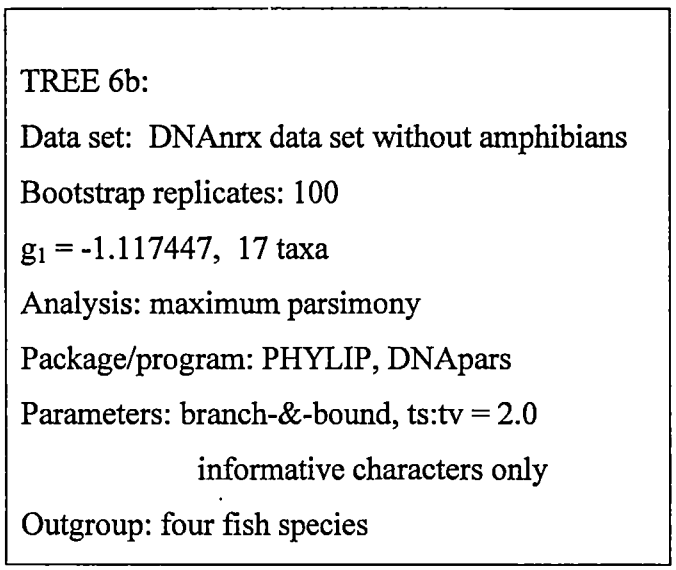

pike
loach
cod
perch
mouse1
monkeya
human1a
mouse2
monkeyb
human2
human4
mouse4
opossumL2
echidnaA
mouse3
human3
chicken
Xenopus
Ambystoma
100
53
50
47
37
30
29
29
96
100
17
65
20
100
100
17
33

Table 5.1 G+C content of vertebrate metallothionein cDNA coding sequences

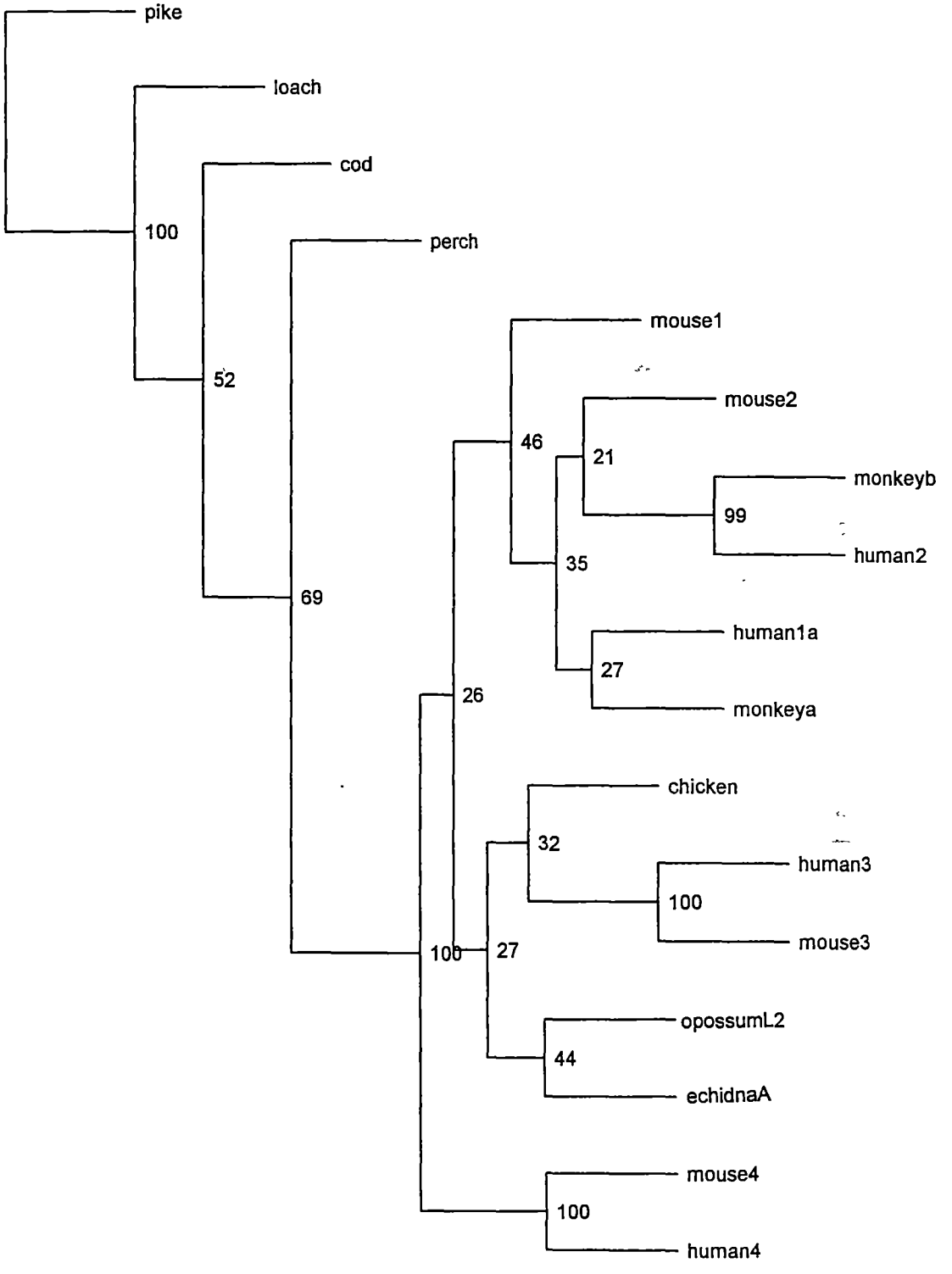
sequence	%G+C content	Genbank accession number	Reference
Ambystoma	53.6	AF008583	Saint-Jacques *et al.* 1997
Bream	59.6	U58774	M. Tom (direct submission)
Chicken	63.5	X06749	Wei & Andrews, 1988
Cod	55.2	U08105	McNamara & Buckley, 1994
Duck	65.6	U34231	Lee *et al.*, 1996
Echidna A	58.9		this thesis
Echidna B	58.3		" "
Goldfish	55.7	X97271	Kille & Olsson (unpublished - 1996)
Hamster 1 (Chinese)	61.8	J00061	Griffith *et al.*, 1983
Hamster 2 (Chinese)	59.1	J00062	Griffith *et al.*, 1983
Human 1a	58.6	K01383	Richards *et al.*, 1984
Human 1b	60.2	M13484	Heguy *et al.*, 1986
Human 1e	62.4	M10942	Schmidt *et al.*, 1985
Human 1f	60.2	M13003	Varshney *et al.*, 1986
Human 1g	61.3	J03910	Foster *et al.*, 1988
Human 1h	62.4	X64834	Stennard *et al.*, 1994
Human 1r	62.4	X97261	Lambert *et al.*, 1996
Human 1x	60.2	X65607	Stennard *et al.*, 1994
Human 2	62.4	J00271	Karin &Richards, 1982
Human 3	61.4	D13365	Tsuji *et al.*, 1992
Human 4	56.6	U07807	Quaife *et al*, 1994
Icefish A *Chaenocephalus aceratus*	57 9	Z72483	Scudiero *et al.*, 1997
Icefish B *Chionodraco ratrospinosus*	57.4	Z72484	Scudiero *et al.*, 1997
Icefish C *Parachaenichthys charcoti*	55.7	AJ007951	V. Carginale (direct submission)
Loach	52.4	X59393	Kille *et al.*, 1991
Monkey A	60.2	V01533	Schmidt & Hamer, 1983
Monkey B	60.8	V01532	Schmidt & Hamer, 1983
Mouse 1	63.4	J00605	Durnam *et al.*, 1980
Mouse 2	59.1	K02236	Searle *et al.*, 1984
Mouse 3	58.5	S72046	Naruse *et al.*, 1994
Mouse 4	56.6	U07808	Quaife *et al.*, 1994

Muscovy	65.6	U34230	Lee *et al*, 1996
Opossum B1	57.7		this thesis
Opossum B3	58.7		" "
Opossum L2	55.5		" "
Opossum L3	57.1		" "
Opossum x5	55.5		" "
Perch	60.7	X97272	Kille & Olssen (unpublished 1996)
Pig	66.1	M29515	Buchman *et al.*, 1989
Pike	50.8	X59392	Kille *et al.*, 1991
Rabbit 1	61.3	X07790	Tam *et al.*, 1988
Rat1	62.9	M11792	Andersen *et al.*, 1986
Rat 2	58.6	M11794	Andersen *et al.*, 1986
Rat 3	56.7	S65838	Kobayashi *et al.*, 1993
Sheep 1a	65.6	X04626	Peterson & Mercer, 1986
Sheep 1c	65.1	X07974	Peterson *et al.*, 1988
Sheep 2	62.4	X07975	Peterson *et al.*, 1988
Trout b	50.8	M22487	Zafarullah *et al.*, 1988
Xenopus	51.9	M96729	Saint-Jacques & Seguin, 1993

Figure 5.9



Tree 6b was calculated with the same parameters as Tree 6a, on a reduced data set from which the amphibian sequences, *Xenopus* and *Ambystoma*, have been omitted.


pike
loach
cod
100
perch
mouse1
52
mouse2
46
21
monkeyb
99
human2
35
69
human1a
27
26
monkeya
chicken
32
human3
100
mouse3
100
27
opossumL2
44
echidnaA
mouse4
100
human4

compositional bias, protein-based and modifed DNA-based data sets were analysed.

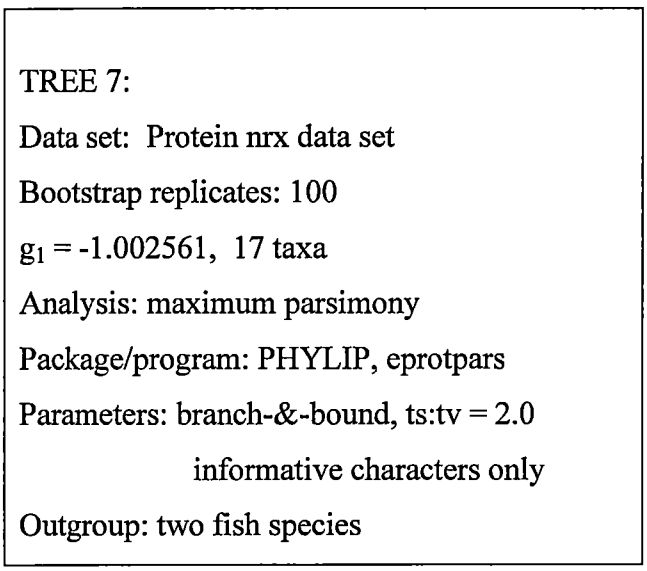
Parsimony analysis applied to comparable extended, non-redundant (nrx) protein sequences, using the PHYLIP **eprotpars** program, gives rise to TREE 7 (Fig. 5.10). MT4 is the ancestral mammalian isoform, with bootstrap support of P=99.7. MT1 and MT2 isoforms constitute a monophyletic grouping (P=53.2). Eutherian MT3, marsupial and bird metallothioneins cluster in a monophyly (P=53.2). This tree is similarto the DNA-based tree in gross morphology, after removal of species believed to introduce distortion due to base compositional bias - TREE6b.

The effects of base compositional bias are exerted predominantly at the third codon position in protein-coding genes. Therefore, third positions of each codon in 100 bootstrapped replicates of the DNAnrx data set were effectively removed by down-weighting, to counter both the problem of G+C disparities and the effects of substitutional saturation at these sites. (The practice of character weighting in phylogenetic analysis is discussed in section A5.5). TREE 8 was computed with the same parameters as TREE 6a on the modified data set. The consensus TREE 8 (Fig. 5.11) places *MT4* as ancestral to all the mammalian *MT*s with 100% bootstrap support. The *MT1* and *MT2* sequences constitute a monophyletic cluster to the exclusion of all other mammalian metallothioneins (P=27). The *MT3*s are ancestral to the marsupial and monotreme metallothioneins, and to the *MT1+MT2* clade. This weighting corrects much of the base compositional bias and third codon saturation in the data set, without recourse to the different set of taxa in the amino acid analysis. The contribution of G+C bias to inconsistencies in the most parsimonious trees is thereby confirmed. The two trees (TREES 7 and 8) are not identical, especially regarding the placement of the amphibia and birds, but the relationships among the mammalian isoforms are consistent.

Summary of Parsimony Analysis

Differences in base composition between taxa affect the calculation of a vertebrate MT phylogeny when DNA sequences are analysed without modification. To circumvent these effects, protein and modified DNA data sets

Figure 5.10



Tree 7 was constructed using the maximum parsimony optimality criterion and the branch-and-bound algorithm, on the informative characters only of the extended non-redundant protein data set (with two fish species removed). Sequence gaps were treated as "misssing" data. No weighting was applied to characters or sites. The tree was rooted on the two fish species. Bootstrap = 100 replicates. A modified 50% majority rule consensus tree was calculated.

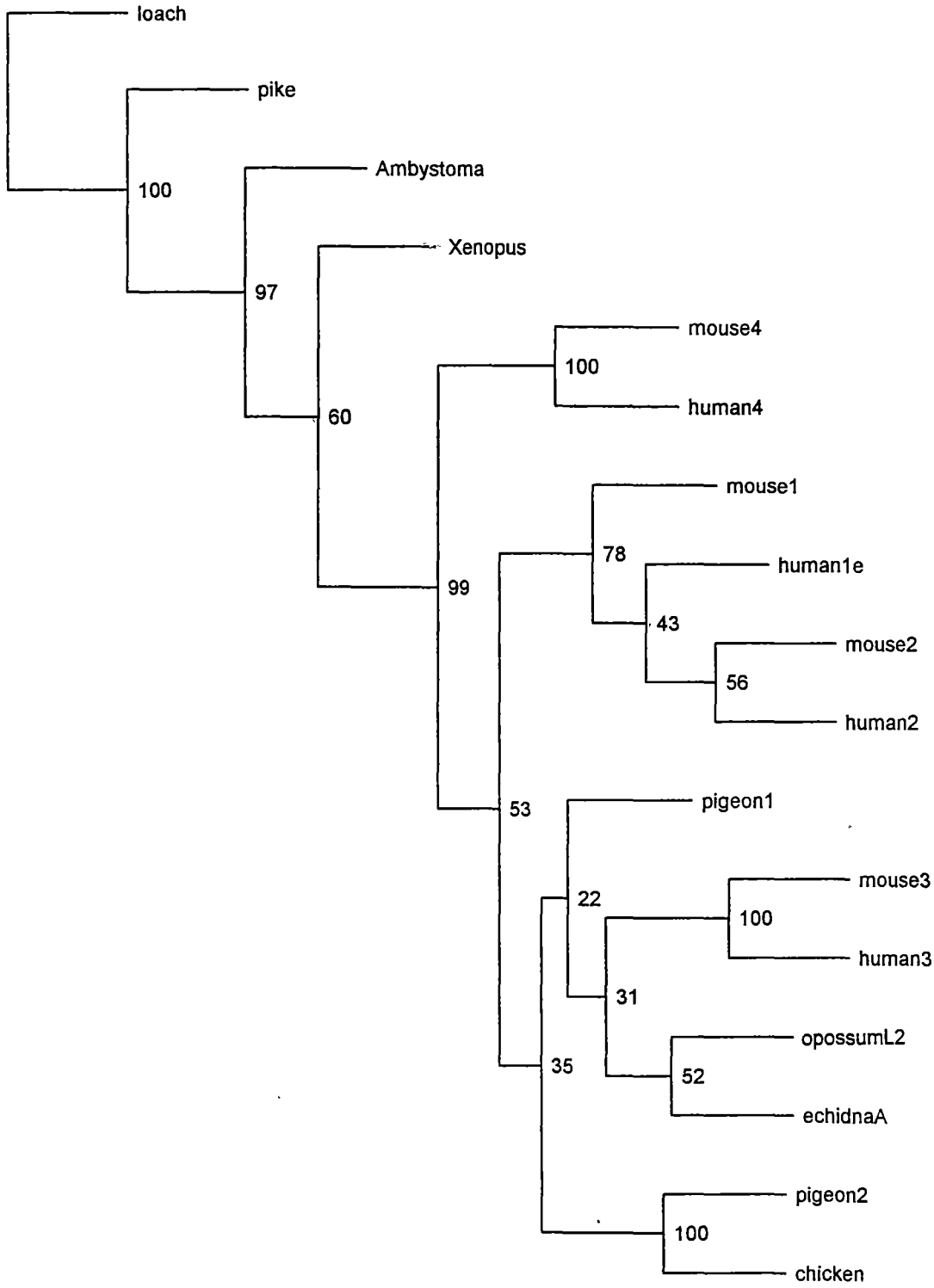
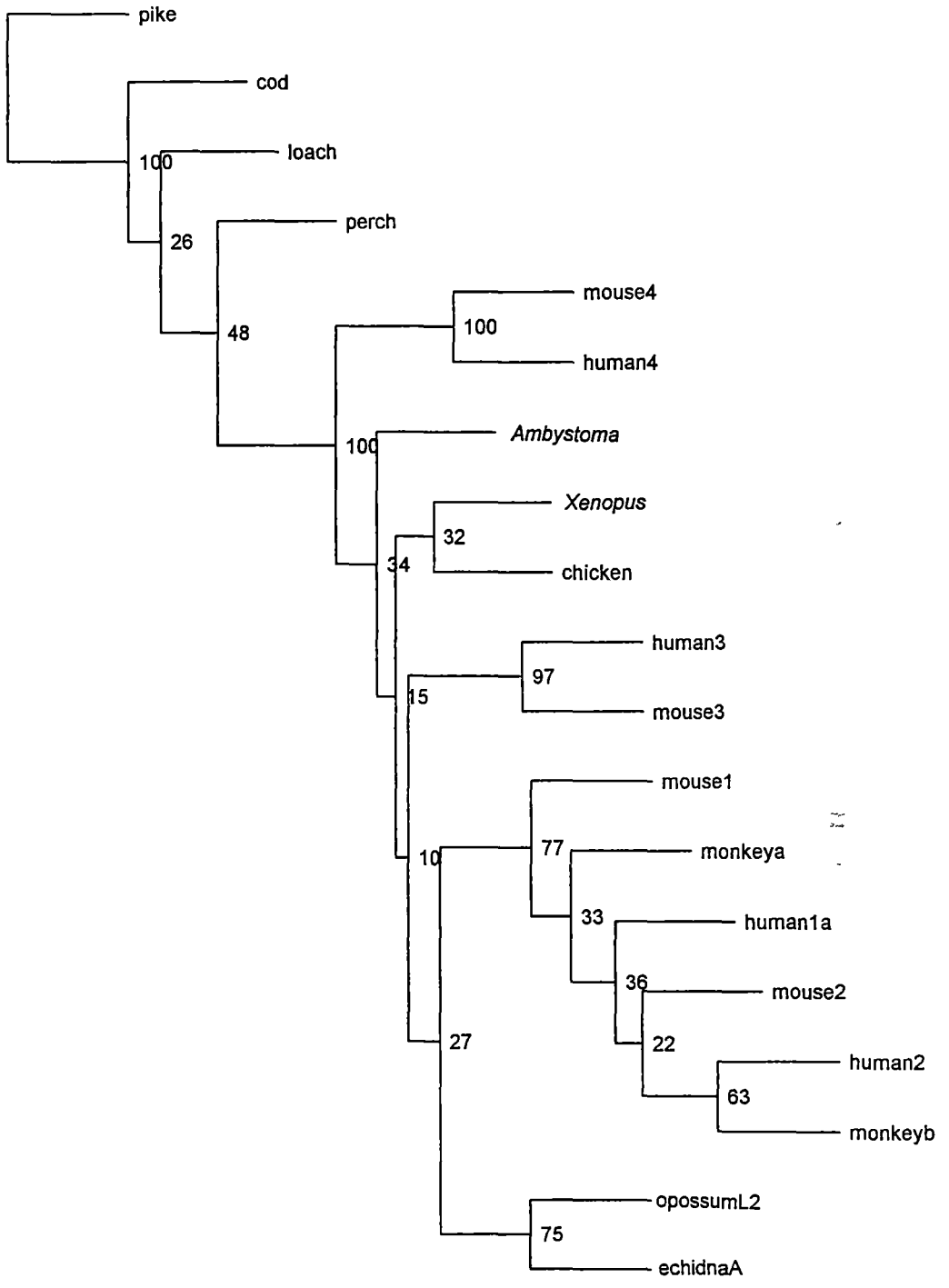

loach
pike
100
Ambystoma
Xenopus
97
60
mouse4
100
human4
mouse1
78
human1e
99
43
mouse2
56
human2
53
pigeon1
22
mouse3
100
human3
31
opossumL2
35
52
echidnaA
pigeon2
100
chicken

Figure 5.11

TREE 8:
Data set: DNAnrx data set
Bootstrap replicates: 100
g_1 = -0.941108, 19 taxa
Analysis: maximum parsimony
Package/program: PHYLIP, ednapars
Parameters: branch-&-bound, ts:tv = 2.0
informative characters only
third codon position = 0
Outgroup: four fish species

Tree 8 was calculated from 100 bootstrap replicates of the extended non-redundant DNA data set under the maximum parsimony optimality criterion, using the branch-&-bound algorithm on informative sites only (randomised order of input). Gaps were treated as "missing". The third codon position was down-weighted to zero to remove it from the computation. The consensus tree was rooted *a posteriori* on the four fish species. A modified 50% majority rule consensus tree was calculated.

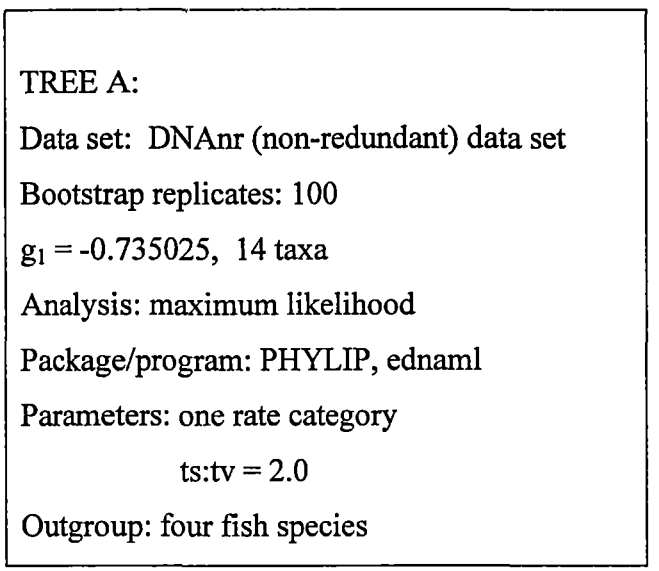

pike
cod
loach
100
perch
26
48
mouse4
100
human4
Ambystoma
100
Xenopus
32
34
chicken
human3
97
15
mouse3
mouse1
77
monkeya
10
33
human1a
36
mouse2
27
22
human2
63
monkeyb
opossumL2
75
echidnaA

were analysed. Removal of the third codon position from the analysis leaves MT4 as the most ancient of the mammalian isoforms, with high bootstrap values. Protein-based reconstructions also place MT4 as the ancestral isoform. Eutherian MT1 and MT2 unfailingly form a monophyletic group to the exclusion of all other MTs, in protein- and DNA-based reconstructions. The monotreme and marsupial clade is consistently monophyletic, and clusters with the eutherian MT3s under most parameters. When the phylogeny is based on the first two codon members only of the DNA sequences, however, marsupial and monotreme *MT*s form a sister clade with eutherian *MT1* and *MT2*.

5.3.2.3 Maximum Likelihood

ML performed on the bootstrapped DNAnr and DNAnrx data sets gave rise to TREE A (Fig. 5.12) and TREE B (Fig. 5.13), respectively. In both trees, *MT3* is the mammalian sequence ancestral to all other mammaliam MTs. *MT4* clusters as sister group to the *MT1* + *MT2* clade. Bootstrap support for virtually all branches of maximum likelihood consensus trees is low. Because different sites along the molecule are substituted at different rates, and with different probabilities (section A5.1.3), the capacity of the DNAML program to specify these parameters was exploited. Four categories were initially defined with substitution rates = 0, 1.0, 0.8 and 2.4 and (arbitrarily chosen) corresponding probabilities = 0.4, 0.2, 0.2 and 0.2, respectively. The consequent TREE C (Fig. 5.14) defines *MT3* as the ancestral mammalian *MT* sequence, and places *MT4* in a monophyletic grouping with eutherian *MT2*. Sensitivity to size of the data set is obvious when comparing the analyses of DNAnr and DNAnrx data sets, which conforms to the empirical demonstration by Lecointre *et al.*, (1993) (and the theoretical confirmation by S. Poe, [1998]) that data sets with fewer than 24 taxa are sensitive to species sampling. Therefore, the analysis was repeated on a larger data set of 33 taxa, taken from the complete data set (TREE D - Fig. 5.15). Various categories of substitution rates and probabilites were tested, with ts:tv ratios of both 2.0 and 20.0, to establish the conditions under which the likelihood of this tree is maximized. All permutations of category number, rates and probabilities produced a tree topologically identical to TREE D. (TREE D,

Figure 5. 12



Trees A and B (Fig. 5.13) were constructed from 100 bootstrap replicates of the non-redundant and nrx data set respectively, using the program DNAML. Ts:tv=2.0. Empirical base frequencies were used, with one category of substitution rates. Global rearrangements were applied, with randomized order of input. Trees were rooted on the four fish species. Trees were reduced to a modified 50% majority rule tree.

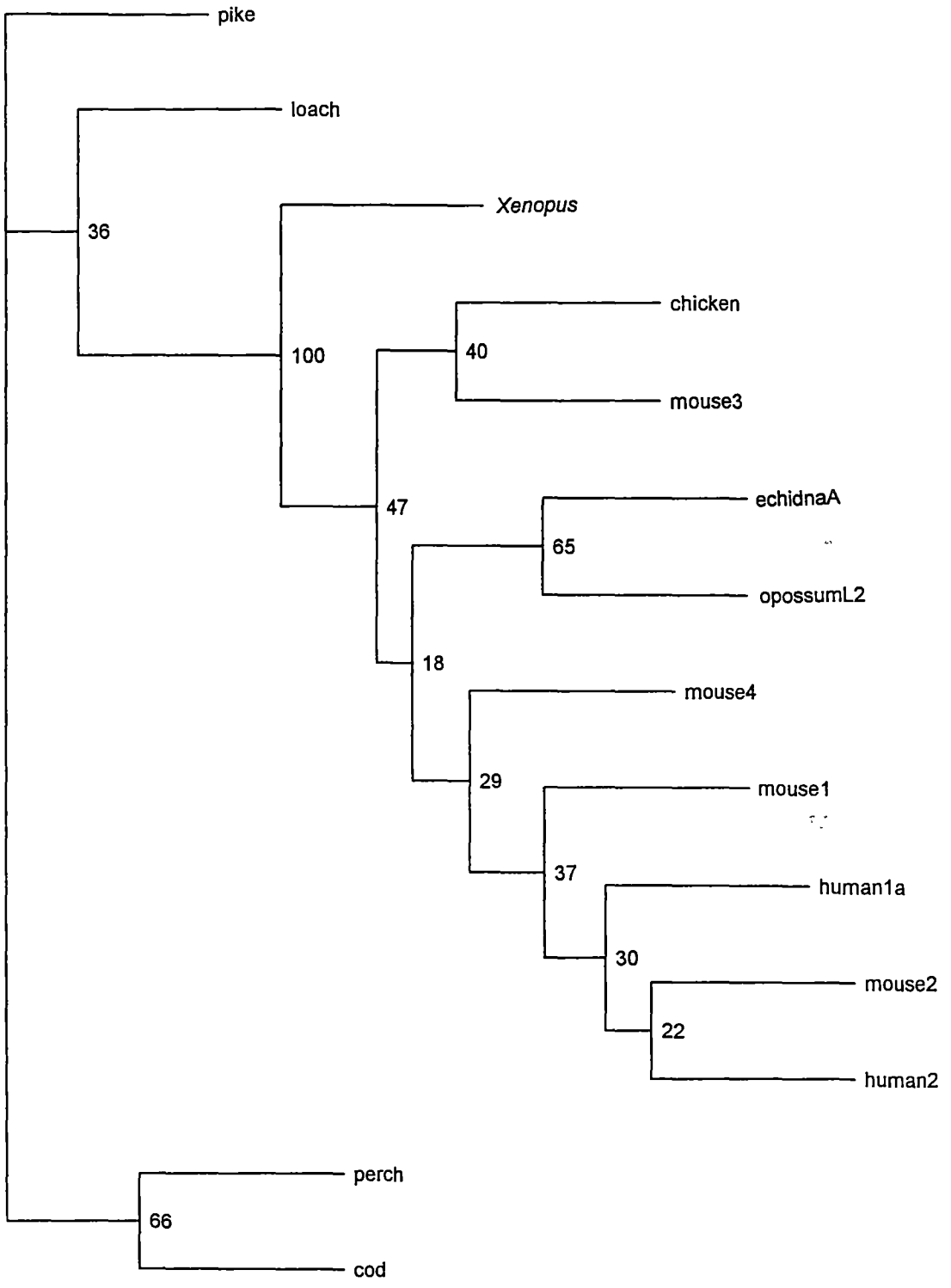
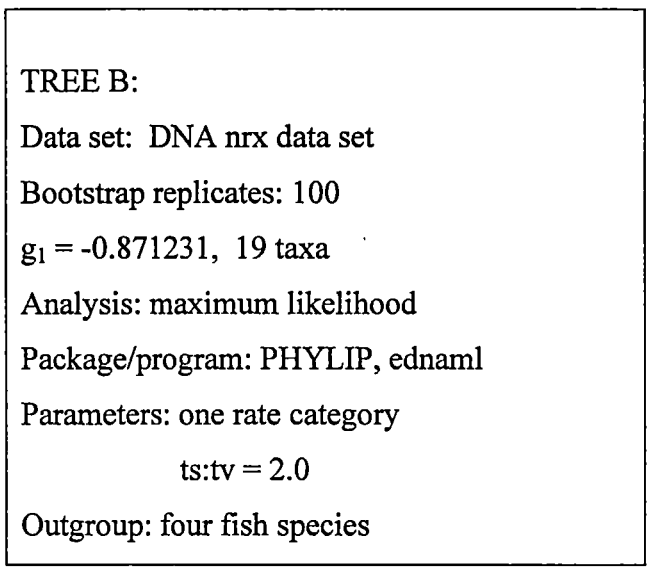

pike
loach
Xenopus
36
chicken
100
40
mouse3
47
echidnaA
65
opossumL2
18
mouse4
29
mouse1
37
human1a
30
mouse2
22
human2
perch
66
cod

Figure 5.13


TREE B:
Data set: DNA nrx data set
Bootstrap replicates: 100
g_1 = -0.871231, 19 taxa
Analysis: maximum likelihood
Package/program: PHYLIP, ednaml
Parameters: one rate category
ts:tv = 2.0
Outgroup: four fish species

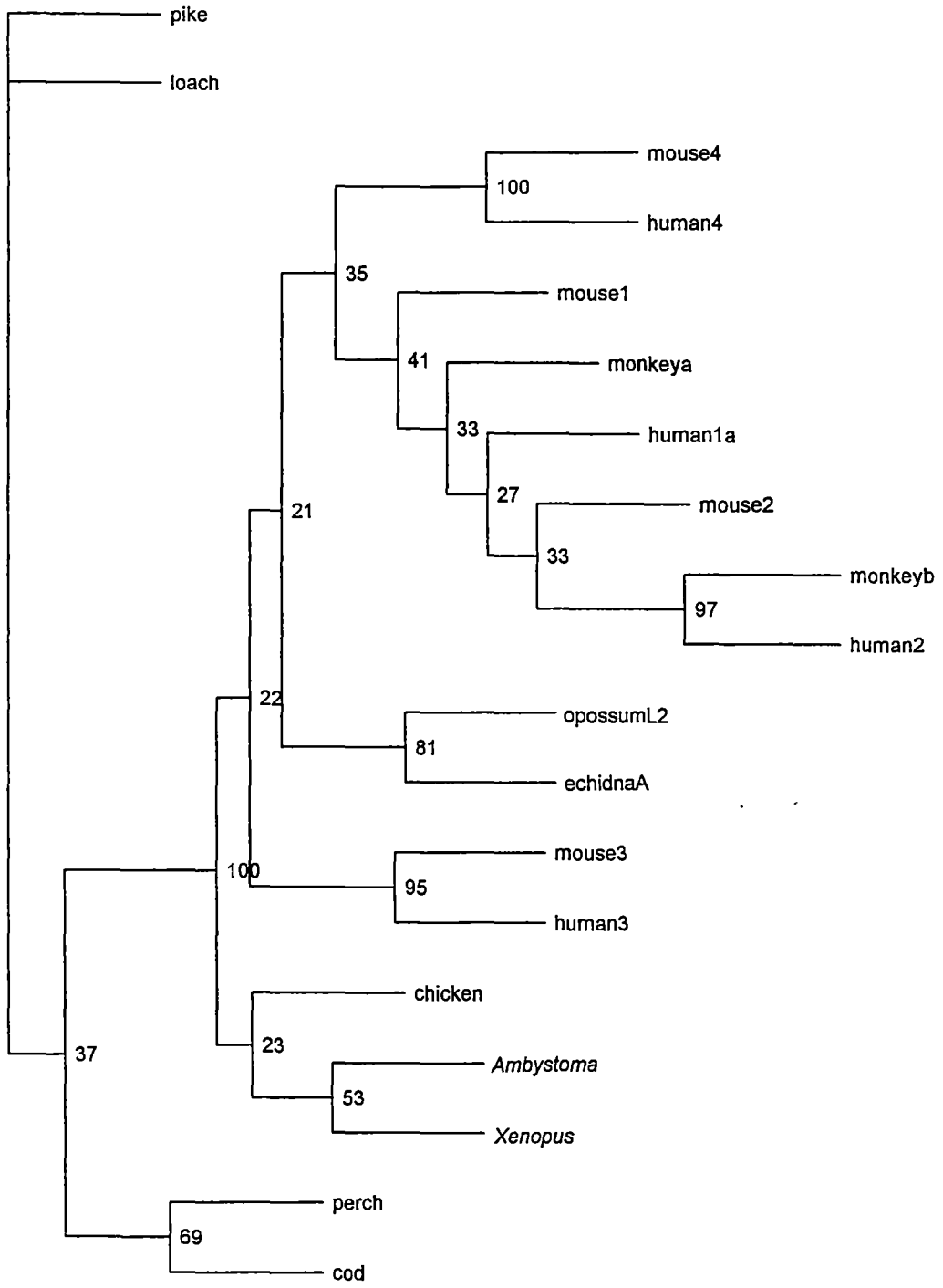
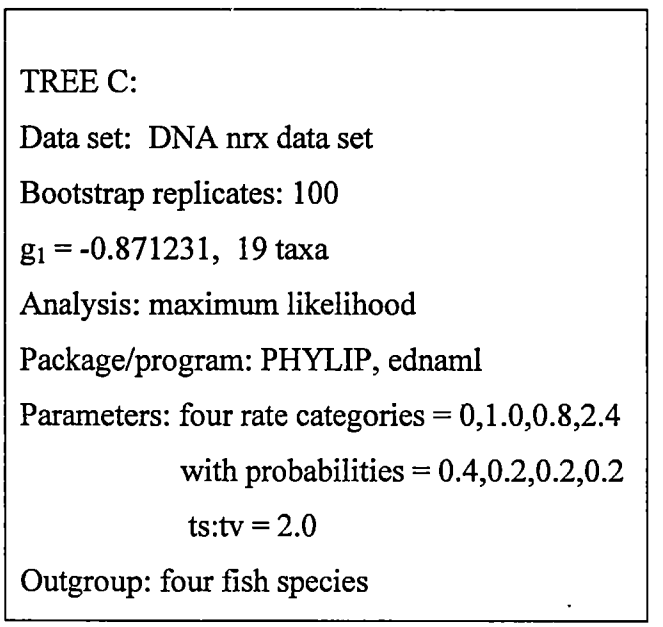

pike
loach
mouse4
100
human4
35
mouse1
41
monkeya
33
human1a
27
mouse2
21
33
monkeyb
97
human2
22
opossumL2
81
echidnaA
100
mouse3
95
human3
chicken
23
Ambystoma
53
Xenopus
37
perch
69
cod

Figure 5.14



Tree C was constructed from the nrx data set, using the program DNAML. Ts:tv=2.0. Empirical base frequencies were used and four categories of substitution rates = 0, 1.0, 0.8 and 2.4 with probabilities 0.4, 0.2, 0.2 and 0.2, respectively. Global rearrangements were applied, with randomized order of input. The tree was rooted on the four fish species.

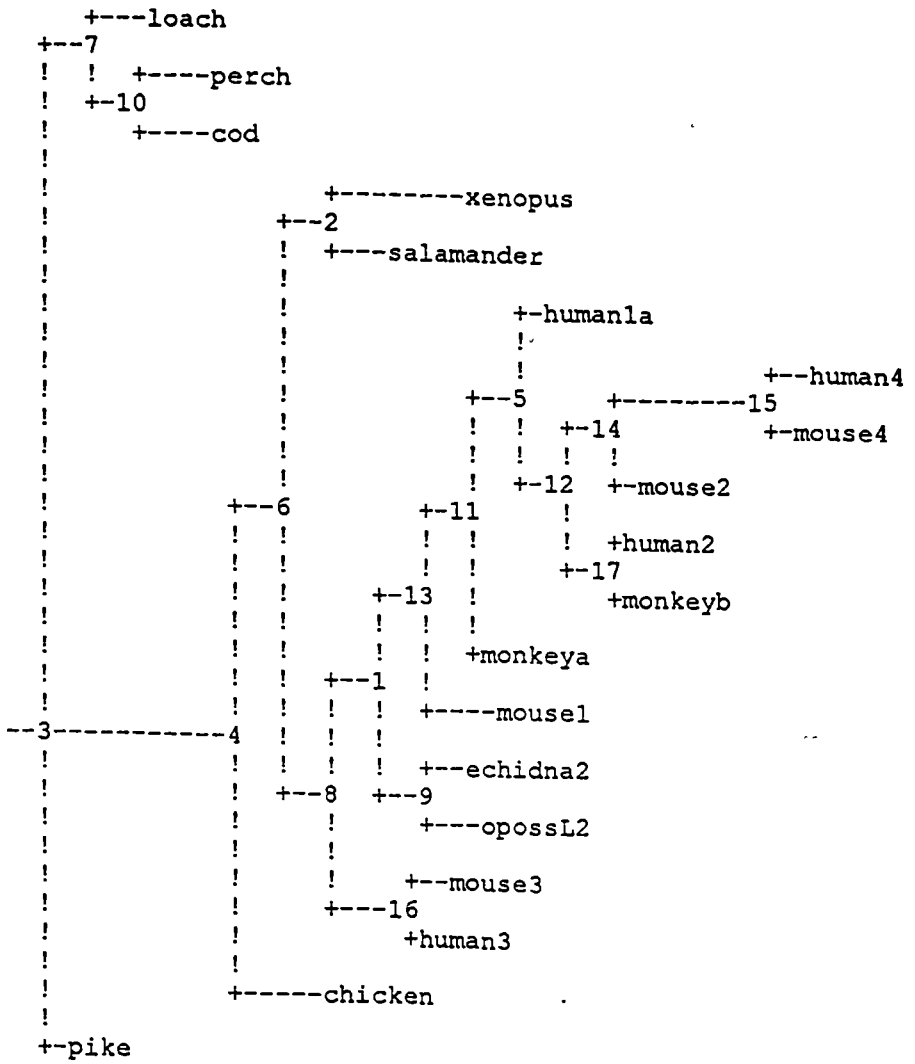
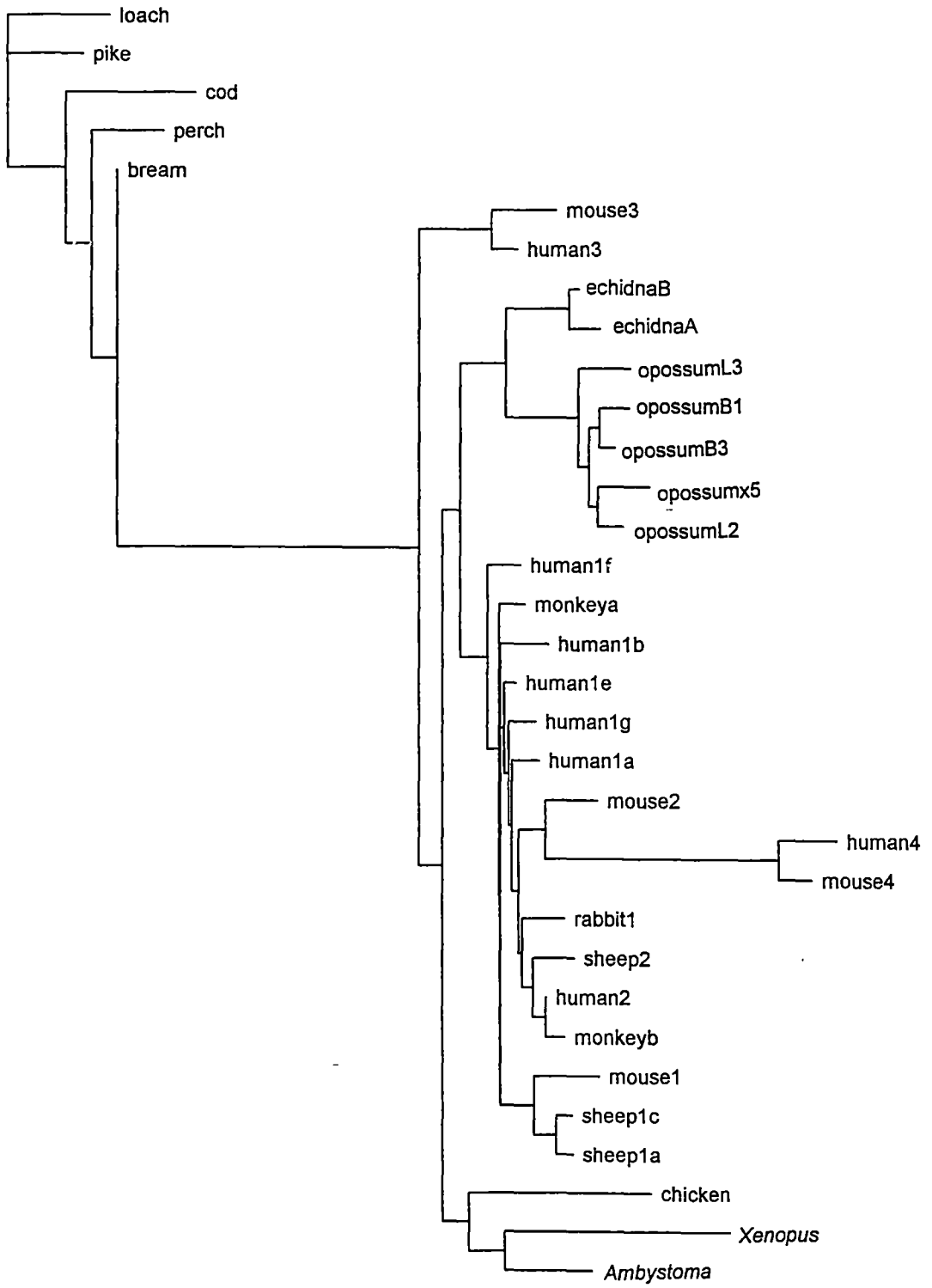

loach
7
perch
10
cod
xenopus
2
salamander
human1a
human4
5
15
14
mouse4
mouse2
12
6
11
human2
17
monkeyb
13
monkeya
1
mouse1
3
4
echidna2
8
9
opossL2
mouse3
16
human3
chicken
pike

Figure 5.15

TREE D:
Data set: DNA nrx data set + extra taxa
Bootstrap replicates: 0
g_1 = -0.569194, 33 taxa
Analysis: maximum likelihood
Package/program: PHYLIP, ednaml
Parameters: four rate categories = 0,1.0,0.8,2.4
with probabilities = 0.4,0.2,0.2,0.2
ts:tv = 2.0
Outgroup: four fish species

Tree D was constructed from an extended data set containing 33 taxa, using the program DNAML and the parameters described for Tree C.


loach
pike
cod
perch
bream
mouse3
human3
echidnaB
echidnaA
opossumL3
opossumB1
opossumB3
opossumx5
opossumL2
human1f
monkeya
human1b
human1e
human1g
human1a
mouse2
human4
mouse4
rabbit1
sheep2
human2
monkeyb
mouse1
sheep1c
sheep1a
chicken
Xenopus
Ambystoma

however, maximises the likelihood of the data set and is therefore the preferred reconstruction of those tested). These trees all place *MT4* as a recent derivative of eutherian *MT2* and evolving rapidly, giving rise to an attendant long branch. Because the computing time is prohibitive, bootstrap support for the larger data set has not been calculated.

ML analysis on a modified non-redundant protein data set produced TREE E (Fig. 5.16) as the most likely tree. TREE E conforms to the protein-based tree calculated by parsimony methods (TREE 7) insofar as the representative MT4 isoform clusters with pigeon MT1. This group forms a clade with the mammalian MT3 isoforms within a grouping that completely excludes the mammalian MT1s and MT2s. TREE E, therefore, corroborates the clustering of mammalian MT4 and pigeon MT1, and their close proximity to MT3 and non-eutherian metallothioneins. Importantly, this is an unrooted tree so the derivations of the various clades cannot be established, only the clustering pattern.

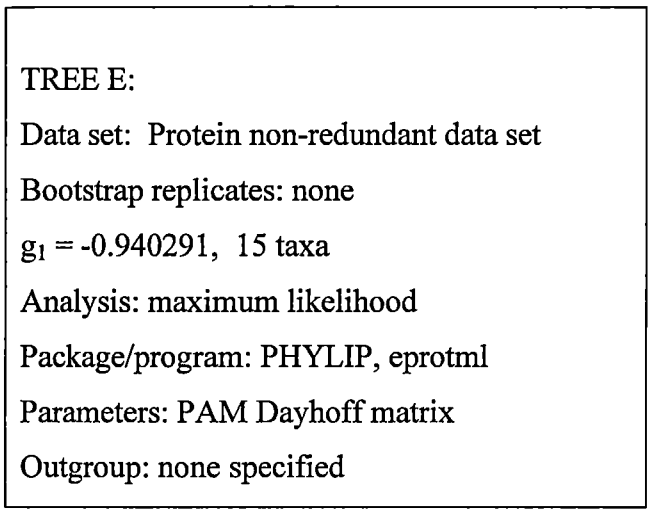
Summary of ML Analysis

When bootstrap replicates of the DNA data sets were analysed with ML, consensus trees placed *MT4* as derivative of mammalian *MT2*. Significantly, however, the bootstrap replicate with the highest log likelihood value in calculations giving rise to trees A and B, placed *MT4* as the sequence ancestral to other mammalian, marsupial and bird *MT*s (tree not shown). Maximum likelihood analysis of the modified non-redundant protein data set placed mammalian MT4 in a monophyly with pigeon MT1, and clustered the clade with mammalian MT3s, and bird and marsupial MTs, contradicting the *MT4* placement of the DNA ML consensus trees.

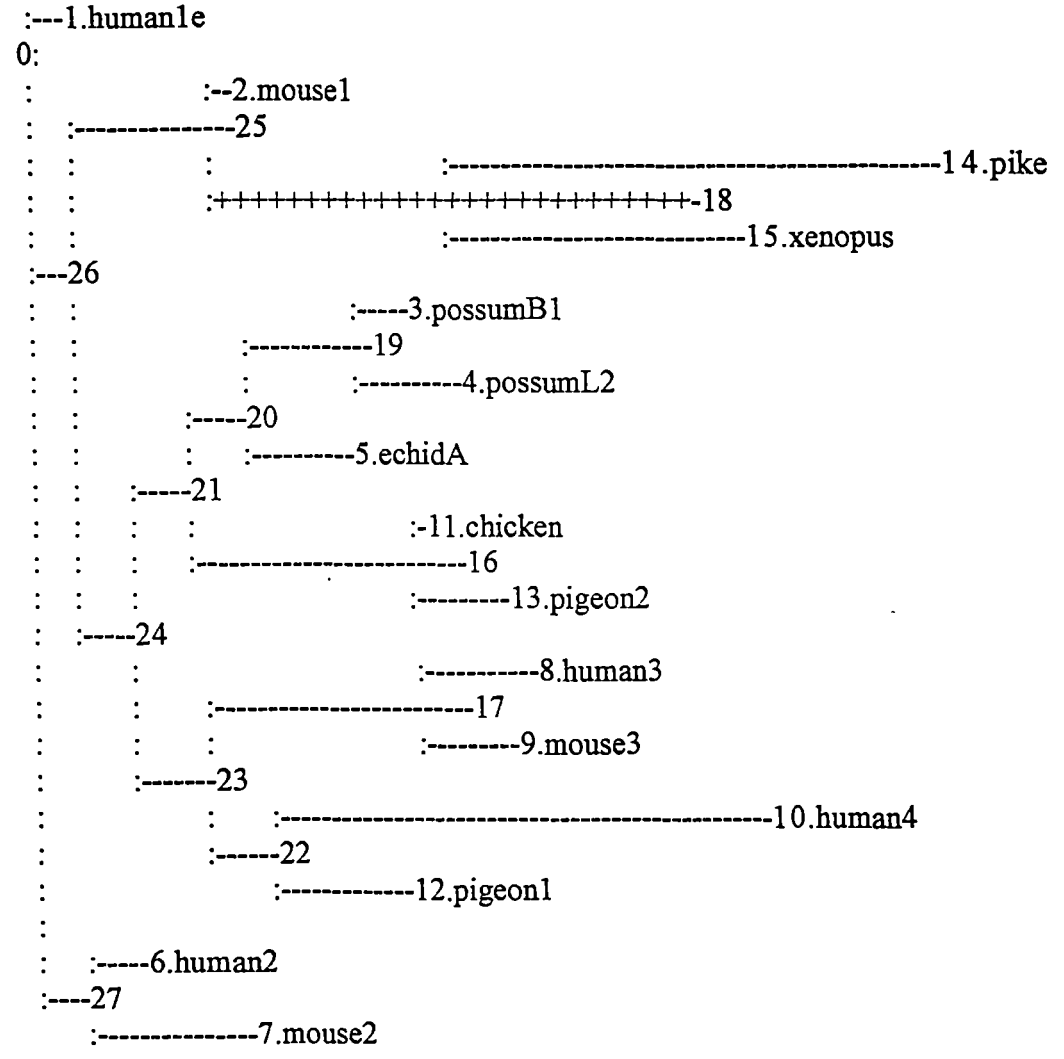
5.3.2.4 Problems with the Bootstrap

Bootstrap values are often very low in distance matrix, parsimony and maximum likelihood analyses of these metallothioneins. Inconclusive bootstrap proportions cannot be attributed to inadequate phylogenetic signal as each data set was described by a g_1 statistic clearly reflecting non-random structure in the data. It is unlikely that low bootstrap values are the result of inadequate taxon

Figure 5.16



Tree E was constructed from a modified non-redundant protein data set with the program PROTML with default parameters. The tree is unrooted.


1.human1e
2.mouse1
25
14.pike
18
15.xenopus
26
3.possumB1
19
4.possumL2
20
5.echidA
21
11.chicken
16
13.pigeon2
24
8.human3
17
9.mouse3
23
10.human4
22
12.pigeon1
6.human2
27
7.mouse2

sampling, as even the complete data sets provided little bootstrap support for many or most of the branchings in distance-based trees. It is recognised that the robustness of a clade is proportional to the number of informative sites used in its calculation (Lecointre *et al.*, 1994), so a small and highly conserved molecule such as metallothionein, with few informative sites available for analysis, will sustain less resolution within a phylogenetic reconstruction than would a longer, less conserved molecule. For instance, Charleston *et al.* (1994) have shown that a sequence length of >180 nucleotides is required in parsimony analysis to infer the correct tree for 10 taxa in at least 70% of simulations, when the data have been corrected for superimposed substitutions using spectral analysis. (This correction of the data is immaterial here, as it does not improve the outcome at sequence length of <1000 nucleotides). Longer sequence is required as the number of taxa increases. Data sets used in analyses here contain >10 taxa and many sites are uninformative. The number of informative nucleotides will, therefore, fall well below the ideal 180. On this basis, it is predictable that resampling from the underlying population using the bootstrap will result in a high proportion of "incorrect" trees and therefore an inconclusive bootstrap value.

The relationships within many mammalian *MT1* and *MT2* genes is obscured by post-duplication gene conversion (Schmidt *et al.*, 1985; Hamer, 1986; Peterson *et al.*, 1988). Phylogenetic signal will be lost and bootstrap values at these sites in the tree will be correspondingly unconvincing. Bird and amphibian sequences tend to be supported by low bootstrap values, especially on the DNA-based trees, which is attributable to base compositions in these sequences that deviates markedly from those of other groups. Bootstrap values are high for the opossum and echidna clusters, as well as for the MT3 monophyly and the MT4 monophlyly. These clades can be assumed stable.

The bootstrap, therefore, is only a partially serviceable measure of reliability in assessing the metallothionein gene phylogeny. High bootstrap proportions reflect dependable groupings, whereas low bootstrap values reflect inadequacy in the data. This inadequacy is intrinsic to the gene itself, and cannot be compensated for by more extensive sampling. Congruence between the different methods of reconstruction serves to support inferences of gene history

to some degree. Certain relationships occur repeatedly through all reconstructions and can, therefore, be accepted with some confidence, especially if they are sustained by extrinsic factors such as the underlying species phylogeny and the pattern of occurrence of insertions and deletions. Insertions and deletions were not incorporated into the computation of phylogeny. Analysis of the occurrence of insertions and/or deletions can, therefore, be used as independent evidence for, or corroboration of, a particular pathway of isoform evolution (discussed below in section 5.5.4).

5.3.2.5 Evaluating the phylogenetic analysis of MT genes

The eutherian MT isoforms 1 and 2 are clearly monophyletic, to the exclusion of the metallothioneins of the closely related marsupials and monotremes. Therefore, it can be inferred that the eutherian MT1s and MT2s arose in the lineage leading to the eutherian mammals after they diverged from the ancestors of extant marsupials. A single ancestral gene was duplicated after the Marsupionta-eutherian split, 130-140 million years ago (Jancke *et al.*, 1997), and before the consolidation of the rodent lineage at 115 million years ago. The new gene subsequently duplicated again, and the resulting genes diverged to produce eutherian *MT1*and *MT2*. By comparing sequence change in *MT1* and *MT2* genes with known divergence dates of species, Griffith *et al.* (1983) calculated a duplication event between 45-120 million years ago. A date of 120 million years ago for this duplication satisfies both Griffith's data and the data presented here. Griffith *et al.* (1983) also propose that the *MT1* gene is evolving more rapidly than the *MT2* sequence. Under currently accepted theories of gene duplication (Li, 1983, 1985), this implies that the initial new product of the ancestral duplication was *MT2*, and its subsequent duplication gave rise to *MT1*.

The eutherian MT3 isoforms cluster repeatedly with both the bird metallothioneins and the metallothioniens of marsupials and monotremes, especially in the protein-based trees. The proximity of bird MT and eutherian MT3 suggests that these sequences are orthologous, implying that the predecessor of both occurred in the ancestral species common to the bird and mammalian lineages, some 310 million years ago.

The opossum metallothioneins presented here fall outside the eutherian MT1 and MT2 clade as a monophyletic group in all phylogenetic reconstructions, with strong bootstrap support. It may, therefore, be inferred that this group of genes arose from a single ancestral gene after the evolutionary segregation of the marsupials. The echidna metallothionein sequences also form a monophyly, branching off close to the marsupial lineage. This implies that the metallothioneins of the opossum and the echidna arose from a single gene present in a common ancestor, and diversified into multiple isoforms after the two lineages separated 116-126 million years ago. This supports and is supported by the Marsupionta theory of marsupial evolution (Janke *et al.*, 1997). The clustering of marsupial and monotreme MTs with MT3 in most trees implies that the progenitor of these metallothioneins was an MT3 orthologue. The occurrence of terminal glutamines in all MT3s, echidna MTs and 40% of opossum MTs, and the presence of a β-domain insertion in all these sequences, sustain this premise.

The position of the MT4 isoform is unstable across phylogenetic reconstructions. In all analyses of protein data sets, in DNA distance matrices corrected for base compositional bias and in parsimony analyses of DNA sequences corrected for third codon position saturation, *MT4* occurs either as the ancestral form of all mammalian and bird *MT*s, or clusters with *MT3* and the non-eutherian metallothioneins. ML analyses of DNA data sets, however, place *MT4* as sister group to eutherian *MT1* and *MT2*. When more taxa are added to the ML analyses to reduce species-sensitivity, a monophyly is established between *MT2* and *MT4*. An ambiguity still remains, however, regarding the ML analysis of DNA sequences. When results of bootstrap replication are reduced to a consensus tree, *MT4* maintains a strong sister relationship with *MT1* and *MT2*. However, examination of all bootstrap replicates individually indicates that of these replicates, the tree with the highest log likelihood value supports an ancestral position for the *MT4* genes.

Significantly, phylogenetic trees based on protein sequences place MT4 in a cluster with MT3 and some non-mammalian MTs, and often as the most ancient mammalian isoform. This can be attributed to several factors. Firstly, analysis of protein sequences circumvents the problems of base compositional

bias, substitutional saturation at third codon positions and differences between species in codon usage, all of which are likely to introduce homoplasy into phylogenetic reconstruction of the metallothioneins. When DNA sequences are corrected for these distortions, trees congruent with the protein-based trees are obtained, confirming that these factors are contributing bias to the analyses. A protein-based phylogeny may then be expected to better represent the history of metallothionein evolution than an uncorrected nucleic acid-based phylogeny. Secondly, the pigeon MT isoforms 1 and 2 are included in the protein-based reconstructions but not in the DNA-based reconstructions, as the DNA sequences for these genes have not been determined. The consistently close relationship between the pigeon MT1 isoform and the eutherian MT4s removes the MT4s from proximity to eutherian MT1s and MT2s in a computational sense, but also establishes that an MT4 orthologue existed in the ancestor common to the mammals and the birds. The association between the MT4 and pigeon MT1 isoforms contradicts any placement of MT4 in a grouping with the eutherian MT1 and MT2 isoforms, and these can therefore be attributed to artefacts of the phylogenetic analysis arising from homoplasy. When the DNAnrx data set is modified to exclude third codon positions from consideration, parsimony analysis produces a tree (TREE 8) similar to the protein-based reconstruction, though with a different combination of taxa. In this way it can be demonstrated that the inclusion of isoforms such as pigeon MT1 and MT2 have not introduced sequence-specific effects into the protein-based trees. An ancestral MT4 metallothionein, with its corollaries, constitutes hypothesis 1 (discussed below).

On the other hand, ML analysis is not subject to the disturbances of inconsistency (Kuhner & Felsenstein, 1994; Huelsenbeck, 1995a,b), shows less susceptibitity to bias introduced from variation in base composition (Lockhart *et al.*, 1994), is less vulnerable to sampling errorr and handles short sequences more accurately than do other methods (Kuhner & Felsenstein, 1994). These attributes would suggest that a phylogenetic analysis of the metallothionein gene family based on ML should inspire more confidence than a parsimony- or distance-based phylogeny. ML analysis of DNA sequences defines *MT4* as the most recently evolved metallothionein, derived from a duplication of the *MT2*

locus. This constitutes hypothesis 2 of metallothionein evolution (discussed below). In accepting this analysis (TREEs C & D), the ancestral position of MT4 in the distance- and parsimony-based trees must be construed as an artefact. The high rate of evolution described for *MT4* in its highly derived position in trees C and D, which gives rise to the extended branch to *MT4*, implies that long branch attraction is likely responsible for the consistent misplacement of *MT4* with the primitive sequences in the parsimony-based trees. The recurrent sister relationship between pigeon MT1 and eutherian MT4 in the analyses of protein sequences must also be dismissed as homoplastic convergence, if this maximum likelihood analysis is accepted as producing the more accurate phylogeny.

In the tree produced by ML from metallothionein protein sequences, TREE E, human MT4 and pigeon MT1 form a monophyletic grouping in agreement with the protein trees derived using other methods. It has been suggested (Reeves, 1992; Adachi & Hasegawa, 1992) that even when evolutionary rate differences between the three codon positions in a protein-coding gene are taken into account, codon usage variation between species can still mislead maximum likelihood analysis of nucleic acids (though usually when genetic distances are very large). In this situation, analysis of protein sequences will provide more reliable resolution than analysis of DNA sequences.

5.4 Evolution of the mammalian metallothionein gene family

Two possible evolutionary scenarios can be devised from these data. These are given in Fig. 5.17 a,b (hypothesis 1) and Fig. 5.18 (hypothesis 2).

5.4.1 Hypothesis 1

Hypothesis 1 is outlined in Figs. 5.17a and 5.17b. *MT3* and *MT4* homologues both occur in mammals and birds, and are therefore assumed to have existed in the ancestral species common to the two groups, the "stem reptile". From the reconstructions presented here, the eutherian MT1 and MT2 isoforms seem most likely to have derived from an MT4 precursor. However, it is possible that they arose from the ancestral MT3. Consequently, two versions of hypothesis 1 are presented, which differ only in this regard. The single ancestral form of the mammalian metallothioneins of hypothesis 1 cannot be

Figure 5.17a - Hypothesis 1a

Hypothesis 1 is based on the premise that MT3 and MT4 occurred in the ancestor common to both birds and mammals. These isoforms both contained a single proline in the α-domain and a single residue insert in the β-domain (referred to here as "inserts", relative to mammalian MT1/2). Figure 5.17 describes the premises and corollaries of hypothesis 1.

Bird Lineage

Both genes were transmitted to the bird lineage; *MT4* maintained in the pigeons as the "MT1" isoform expressed in the liver and *MT3* as the pigeon "MT2" isoform, which is the homologue of the MTs found in other bird species. Both inserts have been maintained in the bird isoforms.

Mammal Lineage

Both genes were transmitted to the ancestor common to all mammalian subclasses. The α-domain insert was lost in the MT4 isoform either in this ancestor, or in the early eutherian from which the subsequent eutherian lineage arose.

Eutherians

In the eutherian ancestor, the proline residue insert of the α-domain of MT3 was substituted with a longer sequence, represented in contemporary species as the 4-6 residue C-terminal insertion of MT3. *MT1* and *MT2* genes arose from subsequent duplication of *MT3*. Both the α- and β-domain inserts have been lost in the MT1 and MT2 isoforms, which suggests that they arose from sequential duplications, as represented in Fig. 5.17, rather than each arising separately from the MT3 precursor.

Marsupials and Monotremes

Marsupials and monotremes share a common ancestor, and a common ancestral metallothionein *MT3* gene. From this gene arose the genes characterised in this thesis. The ancestral MT3 sequence maintained the single α-domain proline. This proline may or may not be maintained in the MT4 isoform, depending on where its loss occurred on the earlier branchings.

The β-domain insert of the marsupial metallothioneins must be presumed lost at either of the two points specified.

No loss of either the α- or β-domain inserts has occurred in the echidna metallothioneins. In this regard, they most closely conform to the bird and putative ancestral sequences.

P = presence of proline insert in the α-domain
P? = proline insert may or may not occur in the α-domain

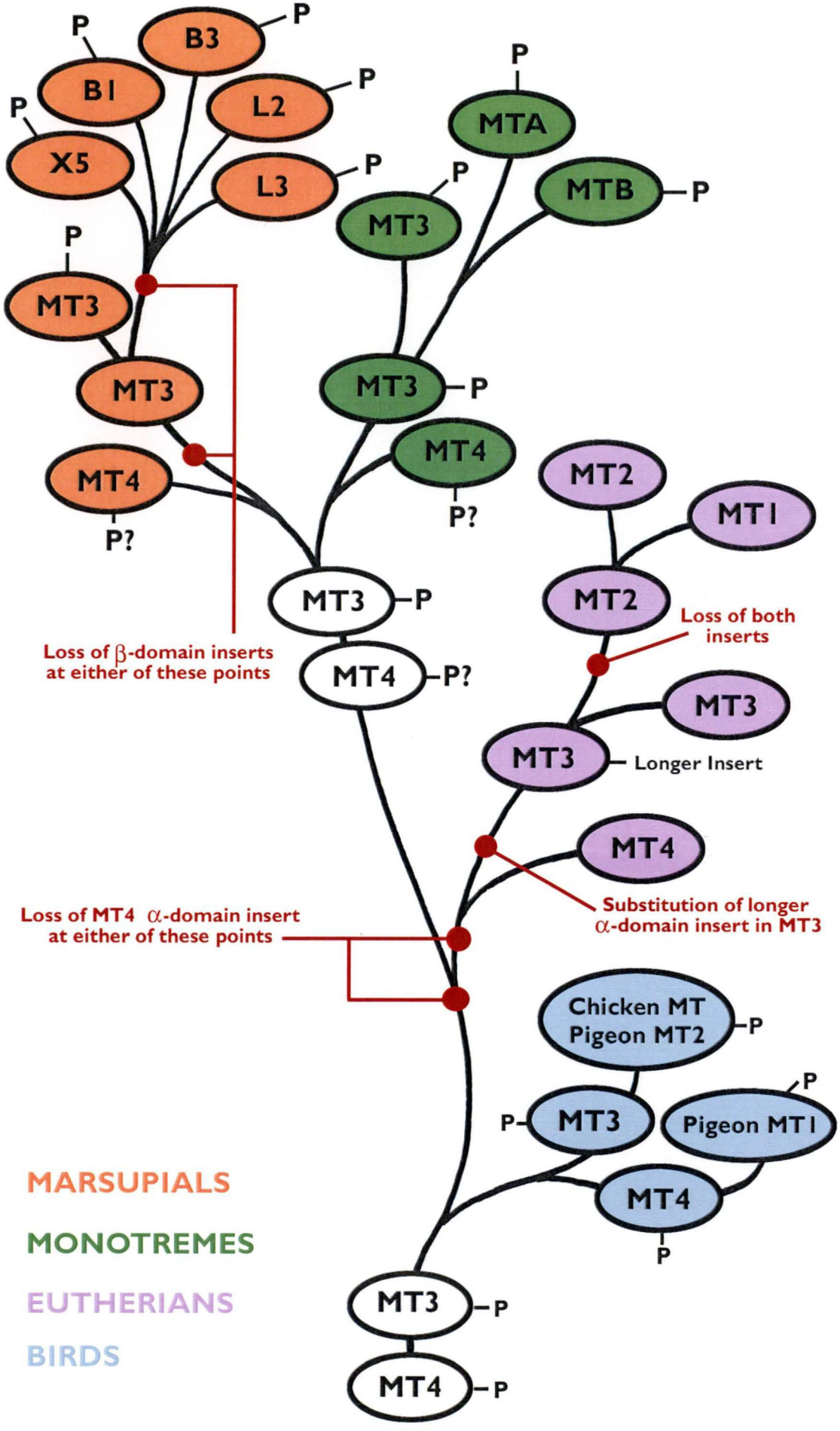
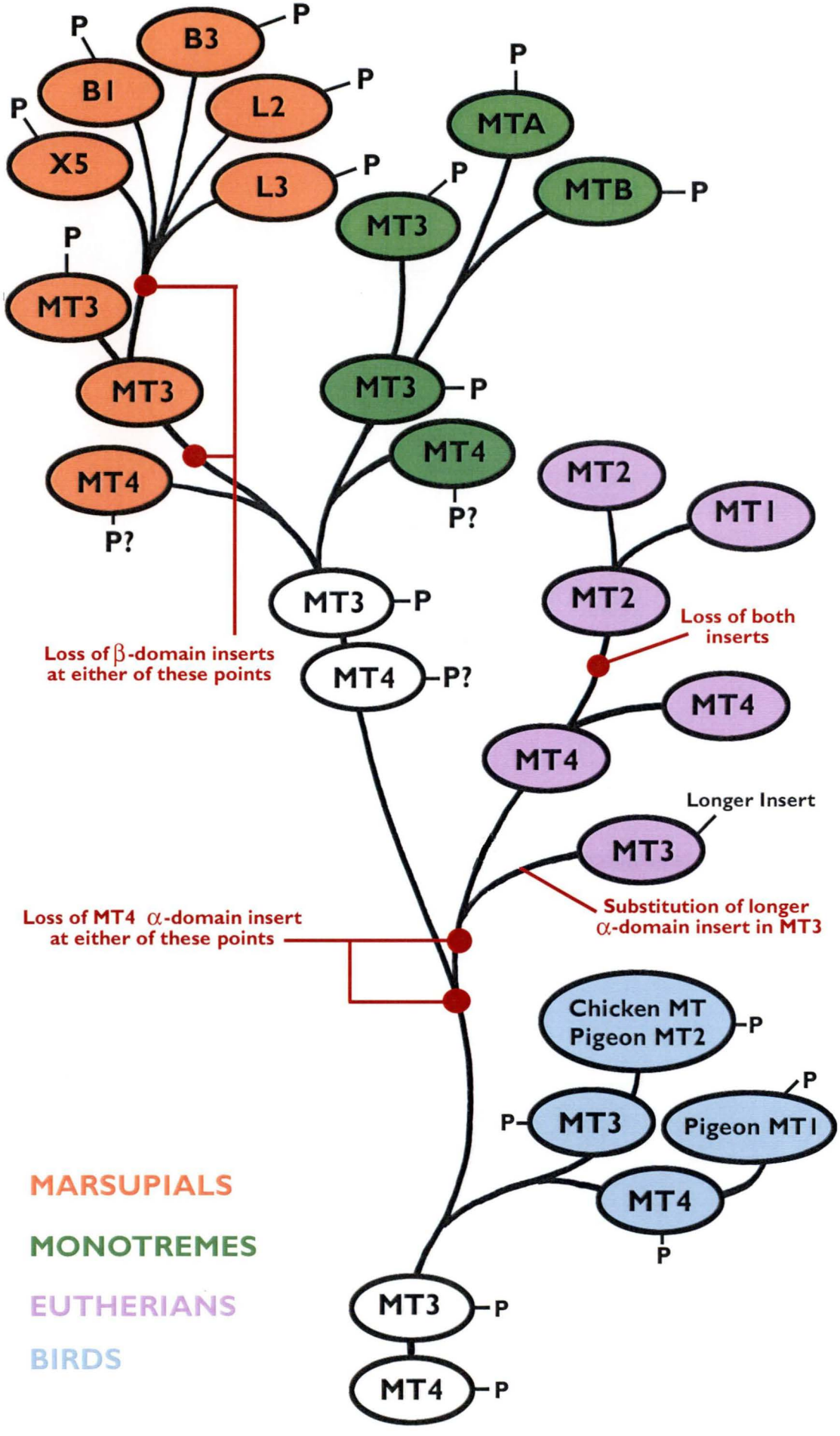

P
B3
P
B1
P
L2
P
X5
L3
P
P
MTA
P
MT3
MTB
P
P
MT3
MT3
MT3
P
MT4
MT4
P?
P?
MT2
MT1
MT3
P
MT2
Loss of both inserts
MT4
P?
MT3
MT3
Longer Insert
MT4
Loss of β-domain inserts at either of these points
Substitution of longer α-domain insert in MT3
Loss of MT4 α-domain insert at either of these points
Chicken MT
Pigeon MT2
P
P
P
MT3
Pigeon MT1
MT4
P
MT3
P
MT4
P
MARSUPIALS
MONOTREMES
EUTHERIANS
BIRDS

Figure 5.17b - Hypothesis 1b

Hypothesis 1b differs from hypothesis 1b only in the derivation of eutherian *MT1* and *MT2* from the the *MT4* gene rather than from the *MT3* gene.

P = presence of proline after residue 52
P? = proline "insert" may or may not occur


P
B3
P
B1
L2
P
P
X5
L3
P
P
MTA
P
MT3
P
MTB
P
P
MT3
MT3
MT3
P
MT4
P?
MT4
P?
MT2
MT1
MT3
P
MT2
Loss of both inserts
Loss of β-domain inserts at either of these points
MT4
P?
MT4
MT4
Longer Insert
MT3
Substitution of longer α-domain insert in MT3
Loss of MT4 α-domain insert at either of these points
Chicken MT
Pigeon MT2
P
P
P
MT3
Pigeon MT1
MT4
P
MT3
P
MT4
P
MARSUPIALS
MONOTREMES
EUTHERIANS
BIRDS

identified with confidence. Consequently, it is prudent to propose the duplication of an unspecified ancestral metallothionein within the stem reptiles (or earlier) giving rise to the ancestral forms of *MT3* and *MT4*.

Hypothesis 1 is supported by the remarkable conservation between the mouse and human *MT4* genes. Where duplication and divergence give rise to new genes, it is understood that the original gene sequence is maintained to support the original function while the second sequence may evolve without the inhibition of selection constraints (Li, 1985; Ohta, 1993). If *MT4* arose from a duplication of the *MT2* locus before the genetic isolation of the order Rodentia, 115 million years ago (as proposed in hypothesis 2), and very soon after the duplication of the *MT2* predecessor producing *MT1*, 115-130 million years ago, and subsequent to the Marsupiontas-eutherian split 130 million years ago, both the *MT1* and *MT4* loci have been freely evolving with minimal selection constraint over a similar period. The mouse and human *MT1* genes have diverged in sequence to a marked degree, and even the more slowly evolving, presumably constrained *MT2* genes of these taxa have similarly diverged. On the other hand, the mouse *MT4* sequence is more similar to the human *MT4* gene than to any other mouse metallothionein (Quaife *et al.*, 1994), and this is vindicated by very high bootstrap proportions in all phylogenetic reconstructions. This suggests that *MT4* has been under strong selection pressure since before the segregation of the mouse and non-mouse eutherian mammals, which is atypical of the recently duplicated copy of a gene (Li, 1985).

Hypothesis 1 predicts the occurrence of *MT4* in the marsupial and monotreme lineages (disregarding the possibility of secondary loss) - see Fig. 5.17a b. Because an MT4 homologue (pigeon MT1) was isolated from the pigeon liver, the tissue specificity attributed to eutherian forms of MT4 need not necessarily pertain in the marsupials and monotremes.

5.4.2 Hypothesis 2

Hypothesis 2 is outlined in Fig. 5.18. The ancestral MT present in the stem reptile gave rise to both the eutherian *MT3* and the bird metallothionein equivalent to pigeon *MT2*. *MT3*, therefore, is the most ancient eutherian metallothionein. *MT4* arose after a duplication of the eutherian *MT2* locus before

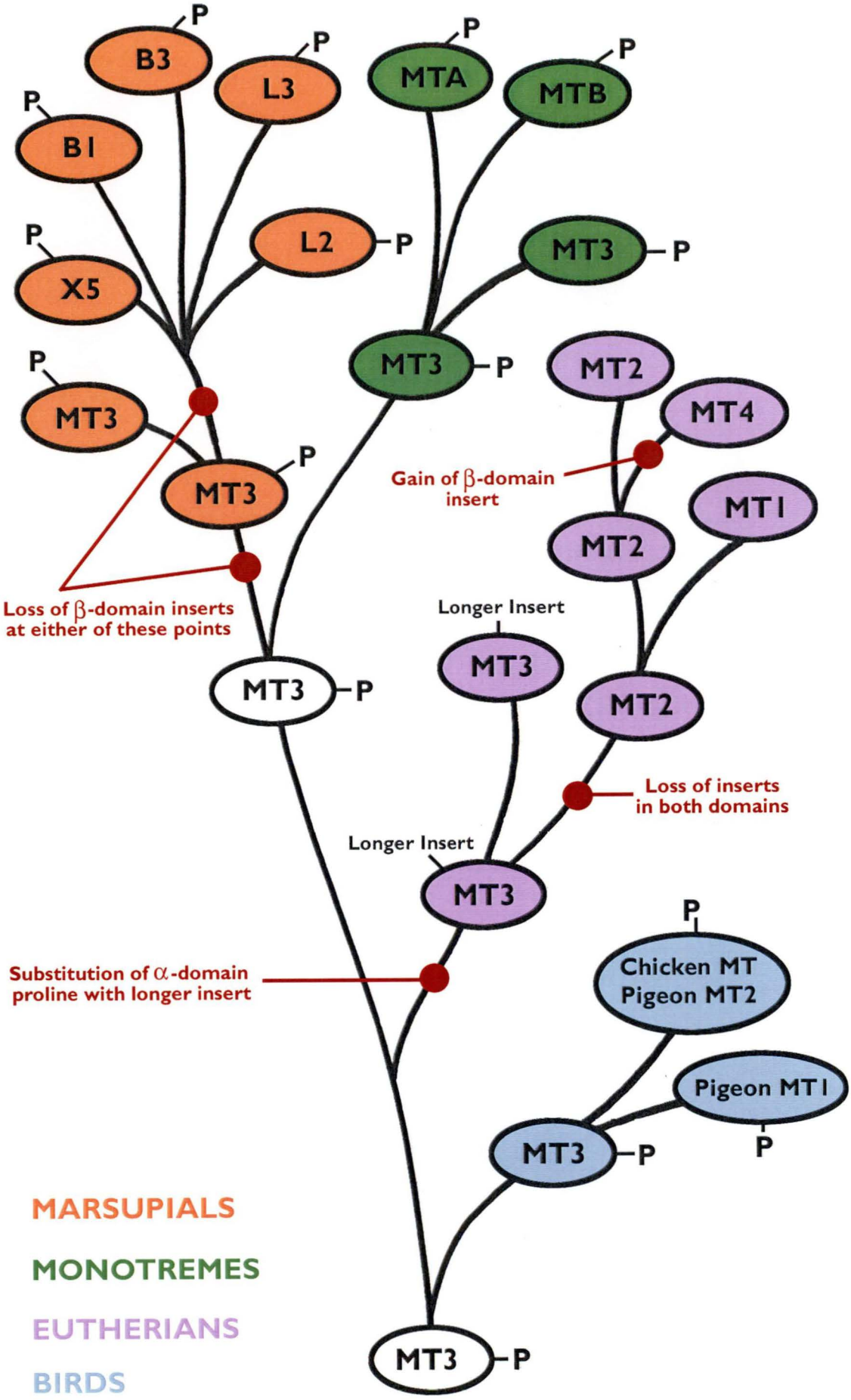
Figure 5.18 - Hypothesis 2

Hypothesis 2 proposes that the bird and mammalian MTs arose from an MT3-like precursor gene. This gene contained the single proline residue "insertion" in the α-domain that occurs in the bird, marsupial and monotreme MT proteins today, as well as the single amino acid "insert" after residue 3 (numbered as in Fig. 3.8). The metallothionein genes of chicken, turkey, pheasant, duck and quail, and pigeon *MT2*, are orthologues of this precursor gene. Pigeon MT1 is the expression product of a duplicated copy of this gene found so far only in the pigeon.

In the lineage leading to the eutherian mammals, the α-domain proline has been substituted with the longer insertion sequence of 4-6 residues, characteristic of eutherian MT3. This gene was duplicated to produce the *MT2* gene, with the loss of both the α- and β-domain inserts. *MT2* was then duplicated to produce the eutherian *MT1* gene. The *MT2* gene duplicated again, giving rise to the MT4 gene, with secondary gain of the β-domain insertion of a single amino acid after residue 3.

MT3 was inherited by both the marsupial and monotreme lineages and was duplicated in each, in ways that are not clear from the available data, to produce the array of genes demonstrated in chapter 3. In the monotremes, both genes retain the proline insert of the α-domain and the single extra amino acid after residue 3 in the β-domain. In the marsupial lineage, the β-domain insert is lost at some point before the occurrence of the derivative genes B1, B3, L2, L3 and x5.

P = presence of proline after residue 52
P? = proline "insert" may or may not occur


B3
L3
MTA
MTB
B1
L2
MT3
X5
MT3
MT3
MT2
MT4
MT3
MT1
MT2
MT3
MT2
MT3
MT3
Chicken MT
Pigeon MT2
Pigeon MT1
MT3
P
Gain of β-domain insert
Loss of β-domain inserts at either of these points
Longer Insert
Loss of inserts in both domains
Longer Insert
Substitution of α-domain proline with longer insert
MARSUPIALS
MONOTREMES
EUTHERIANS
BIRDS

the separation of the rodent lineage, and has evolved rapidly since that time. Hypothesis 2 suggests that no MT4 isoforms will be found in the marsupials or monotremes.

5.4.3 Prediction of *MT3*

Both hypotheses predict the occurrence of MT3 in the opossum and echidna lineages. Hypothesis 2 proposes MT3 as the precursor of all mammalian metallothioneins. Hypothesis 1 states that both MT3 and MT4 occurred in the ancestor to all birds and mammals. As described above, shared protein features and phylogenetic clustering imply the opossum and echidna *MT* genes arose from a duplication of the ancestral *MT3* gene. As the ancestral gene, *MT3* is predicted to be perpetuated after duplication. Furthermore, if MT3 is presumed to have a specialized function that accounts for the attributes by which it can be discriminated from the other MTs, and these attributes are not shared by the five opossum MTs, it is highly likely that the MT3 function is still required by opossums, and the isoform will persist.

5.4.4 Insertions and deletions

Insertions and deletions (indels) can often be significant in the evaluation of gene evolution (eg. Baldauf & Palmer, 1993). There are two principal sites in the metallothionein protein at which indels occur: between residues three and four of the N-terminal β-domain and after residue 52 in the C-terminal α domain, relative to eutherian MT1 and MT2 sequences (Fig. 3.8). The MT3 and MT4 isoforms all have a single residue insertion in the β-domain in common with *Xenopus*, bird and monotreme metallothioneins. Both hypotheses support this insertion as the ancestral form in mammals. The *Ambystoma* sequence has a two residue deletion relative to MTs 1 and 2 at this position (three residues missing relative to the *Xenopus* sequence), and the fish metallothioneins have a one residue deletion (see Appendix II), suggesting that the insertion into the ancestral metallothionein occurred within the amphibia and was secondarily lost within the marsupials and again within the eutherian MT1 and MT2. Hypothesis 2 proposes that the occurrence of this insert in *MT4* is the result of secondary gain after duplication of the *MT2* gene. These insertions and deletions have

occurred with apparent maintenance of function (if it is assumed that the function of the bird and monotreme MTs corresponds to that of the opossum MTs, or that the functions of MT in *Xenopus* and *Ambystoma* are the same), which indicates a certain lability within the protein molecule at this point. Hypothesis 1 is more strongly favoured by the distribution of the insertion in the N–terminal domain through isoforms than is hypothesis 2.

Eutherian MT3 has a distinctive 4-6 residue insertion in the C-terminal domain. Birds, marsupials and monotremes share this insertion as a single proline residue at this site. The most parsimonious explanation of the occurrence of these insertions is a single event in the MT3 orthologue present in the stem reptile from which both mammalian and bird lineages arose. The proposed insertion at this point is a single proline which was subsequently maintained in the bird and marsupial/monotreme lineages. The proline was substituted with a mobile 4-6 residues insertion later in the eutherian lineage. The occurrence of a single proline in the pigeon1 isoform, proposed in hypothesis 1 to be an MT4 homologue, suggests that this may be the ancestral form subsequently lost from MT4 in eutherian mammals. The C-terminal insertion does not occur in eutherian MTs 1 and 2 and is presumed secondarily lost in the common ancestral form of these genes. Hypothesis 2 is well recommended by the occurrence of a single proline residue at this point in both pigeon MTs, as no multiple gain and loss from MT4 need be postulated. The occurrence in the *Ambystoma* MT protein of a lysine at this site could represent an independent insertion event, or suggest that the insertion first arose within the amphibians, mutated to proline in the lineage leading to the birds and mammals and was secondarily lost in other amphibians such as *Xenopus*.

The α-domain insertions found in the opossum and echidna metallothioneins are significant in that they identify these genes as sharing an orthologous relationship with the avian MTs and the eutherian MT3s. Together with phylogenetic analyses, they indicate that the marsupial and monotreme MTs should preferentially be classified with these isoforms, rather than in a grouping with eutherian MT1 and MT2. The β-domain insertion in the monotreme metallothioneins is the primitive form, shared with eutherian MT3 and possibly

with MT4. If or when MT4 is established unambiguously in either the bird, marsupial or monotreme lineages, the significance of this insert will be clarified.

5.5 Conclusions and speculations

Inclusion of the marsupial and monotreme metallothioneins within the phylogenetic analysis of the *MT* genes has allowed recognition of the MT1 and MT2 isoforms as purely eutherian entities, only recently evolved in the history of the gene family. These findings have repercussions for the future classification of the class I metallothioneins.

The occurrence in the mammals of a complex multigene family of metallothioneins is in contradistinction to the markedly simple MT family in the birds. It is interesting to speculate that an attribute of mammalian physiology has required an increase in gene number or in some subtle protein attributes of the metallothioneins that has no counterpart in avian physiology. It is also significant that the three mammalian subclasses appear to have independently undergone multiple duplication of the MT locus within the period after the segregation of the mammals. This suggests the incidence of a stimulus acting upon the mammalian characteristics common to each subclass inducing the same genetic response. It is possible to propose a time for the occurrence of this phenomenon in broad terms, here, from the known dates of lineage separation. The MT1 and MT2 isoforms were established in the Eutheria before the genetic isolation of the Rodentia at 115 million years ago, but after the Marsupionta-eutherian split at 130-143 milion years, approximately 130 million years ago. The metallothionein genes of the monotremes and marsupials were duplicated after the divergence of these taxa, 116-126 million years ago (dates are taken from Jancke *et al.*, 1997). Therefore, it is possible that a "universal", albeit independent, duplication of the *MT* locus may have occurred in each of the mammalian subclasses around 120 million years ago. This is a testable hypothesis. Considerable data are now available on the evolution and phylogenetic diversification of the Marsupialia. It has been demonstrated from DNA-DNA hybridisation data, for instance, that the extant marsupials form a monophyletic grouping arising from a common ancestor after the end of the

Cretaceous, 65 million years ago (Kirsch *et al.*, 1997). Comparison of these sorts of evolutionary parameters with the structure of *MT* gene families in different Marsupionta lineages should allow specification of the interval over which the marsupial and monotreme *MT* genes multiplied.

Human and sheep metallothionein gene families have undergone further subsequent multiple duplication of the *MT1* locus. The rabbit, on the other hand, has undergone duplication of the *MT2* locus. Therefore, it can be inferred that these duplications occurred after the segregation of the rabbit lineage, at 65 (Novacek, 1992) to 90 (Penny & Hasegawa, 1997) million years ago. The pig *MT1* and *MT2* loci both appear to have undergone multiple duplication (Huang *et al.*, 1998). When the full extent of the duplications in various mammalian metallothionein familes is known, it should be possible to establish whether this second round of gene duplication occurred "instantaneously" (in terms of evolutionary time) and if so, whether it was coincident in all taxa. It would be possible, thereby, to establish whether a single environmental trigger was responsible across groups: once again a testable hypothesis.

The evolution of gene families is generally accounted for by the assumption of sequential duplication and divergence of a single ancestral gene. However, I here propose an alternative mechanism for the evolution of the metallothionein gene family. The amplification of the metallothionein locus has been demonstrated in cultured cell lineages, microorganisms and intact mammals in response to sublethal levels of heavy metals (discussed in section 1.1.8). This amplification has been shown to persist after the removal of the inducing agent. It is feasible that a similar inducing agent triggered the amplification of the *MT* locus across mammalian groups in response to some requirement specific to mammalian physiology. If the coincidence of gene multiplication can be established for the mammalian metallothioneins, some such environmental stimulus can be sought. Is such a globally effective environmental phenomenon, capable of triggering such a sweeping consequence, plausible?

The existence of an abnormal concentration of the transition metal, iridium, in worldwide sediments dated at 65 million years ago, has been termed "the iridium anomaly". This anomalous deposit has been imputed to the impact

of a chondritic asteroid with the earth at that time, which enveloped the earth for a considerable period in a fine layer of dust with a very strong component of this metal. A literary corpus exists relating this cataclysm with the mass extinctions at the Cretaceous-Tertiary boundary, which included the loss of the dinosaurs. Alternative explanations of the iridium anomaly in terms of global vulcanism (Officer & Drake, 1983, 1985), for instance, or changes in palaeo-redox conditions (Wang *et al.*, 1993), do not detract from the recognition of a worldwide atmospheric dispersal of high levels of iridium. Iridium occurs in the same column of the periodic table as cobalt; cobalt induces (Bracken & Klaassen, 1987; Rosenberg & Kappas, 1989) and is bound (Vasak *et al.*, 1987) by metallothioneins. Though no work has been reported to this effect, it is likely that iridium itself interacts in a comparable way with metallothionein. It is feasible that some such global phenomenon as the high levels of atmospheric iridium that produced the iridium anomaly, induced an amplification of the metallothionein locus independently and simultaneously in different mammal groups. The initial multiple duplication event proposed here for the mammalian metallothioneins, at around 120 million years ago, is not temporally consistent with the iridium anomaly identified at the Cretaceous-Tertiary boundary. I suggest that the multiple duplication of the eutherian *MT* locus (and possibly the marsupial and monotreme loci) at this time occurred in response to the physiological demands of being a mammal. The second round of amplification of the eutherian locus, typified by the multiple duplication of rabbit *MT2* and human *MT1*, may be consistent with the extraordinary iridium levels of 65 million years ago. (The escape of the muroid rodent species from the effects of this phenomeneon must, necessarily, be accounted for).

The value of this hypothesis is its testability. If it is shown that the number of metallothionein genes increased independently and simultaneously in different lineages of mammals, we can propose a widespread environmental trigger for this increase. Such global phenomena have demonstrably occurred. This trigger has acted only on mammalian physiology and we can, therefore, compare mammalian and non-mammalian physiologies, the better to understand the role of metallothioneins in these two groups, and the general physiological

functions of the metallothioneins in general. Furthermore, there are implications for evolutionary theory:

1. a new mechanism for the evolution of multigene families is proposed;
2. direct environmental impact on heritable traits is implied;
3. a genetic mechanism supporting the theoretical premise of punctuated equilibrium is suggested.

The metallothioneins have lent themselves, over the years, to many and varied biological research applications: from widespread use of the *MT* promoter in genetic engineeering enterprises, to insights into transcriptional control. That they should contribute also to our understanding of the mechanisms of gene family evolution seems appropriate. While the function or functions of the metallothioneins themselves remain elusive, their contribution to our understanding of biological principles continues, ironically, to increase.

Appendix to Chapter 5

The appendix to Chapter 5 provides a synopsis of phylogenetic methods and the parameters upon which they are based. It is especially directed to assist those with little or no background in phylogenetics to follow the methodology and interpretation of Chapter 5. Reciprocal cross-references are made between the text of Chapter 5 and the descriptions of this appendix.

A5.1 Methods of Phylogenetic Inference

A5.1.1 Distance Methods

Distance methods of phylogenetic analysis have two stages. Initially a distance matrix of pairwise dissimilarities between sequences is constructed. These are "uncorrected distances" and are equal to the proportion of sites at which the two sequences are dissimilar (Fitch & Margoliash, 1967). Distances are "transformed" to correct for multiple (or superimposed) substitutions at a single site along the molecule (Felsenstein, 1984).This transformation is a particular advantage of distance measures because it diminishes the impact of homoplasy on the analysis (Olsen., 1988). There are several transformation models that can be applied to nucleic acid data, which are predicated on increasingly complex sets of assumptions.

1. The Jukes-Cantor (JC) model (Jukes & Cantor, 1969) assumes that character state changes at each site in the molecule are mutually independent, and that they occur with equal probability.
2. The Kimura-2-parameter (K-2-P) model (Kimura, 1980) is similar to the JC model except that it allows for differing rates of transitional and transversional changes.
3. The Jim & Nei (JN) model (Jin & Nei, 1990) is a variation of the Kimura-2-parameter model wherein the substitution rate at different sites is allowed to vary, and this variation follows a gamma distribution.
4. The LogDet transformation (Steel, 1994; Lockhart *et al.*, 1994) can be used under the assumptions described for the other models, and is especially useful because it is insensitive to bias in G+C content.

When the assumptions under which a model operates are broad, the less likely is a given data set to violate them (Swofford *et al.*, 1996). For relatively short sequences, JC distances have been shown to give rise to the "true" tree as effectively as any more complex model (Nei, 1991).

Distance matrices drawn from protein sequences may be based on models of amino acid substitution such as the PAM Dayhoff transformation (Dayhoff, 1978), in which an empirical scale of substitution probabilitites for each pair of amino acids is used to compute distances between protein sequences. Alternately, matrices may be based on a combination of amino acid substitution probabilities and a form of the Kimura-2-parameter method applied to the underlying nucleic acid sequence, as in the Categories method (Felsenstein, 1995).

The second stage of the distance methods involves the computation of a tree from the distance matrix. This process is algorithmic, and several algorithms are available including the neighbour-joining (NJ) method of Saitou & Nei (1987) used here. Neighbour-joining is related to cluster analysis insofar as the tree is produced by reiteratively linking the least distant pair of nodes rather that the least distant pair of taxa or clades. A node is the point at which two branches meet. Nodes are calculated from a modified distance matrix and the separation between each pair of nodes, the branch length, is adjusted to allow for different rates of change in different lineages (Felsenstein, 1995). Because the NJ method doesn't assume a molecular clock, an unrooted tree is produced and rooting must be justified on extrinsic criteria.

Distance matrix methods only compare the mean distances between sequences and, therefore, some phylogenetic information is necessarily lost. Furthermore, as with all algorithmic methods, there is the fundamental difficulty that the evolutionary property actually described by branch-length (or genetic distance) cannot be defined (Farris, 1986).

A5.1.2 Parsimony

Parsimony is the most commonly used method of phylogenetic inference (Swofford *et al.*, 1996). It gives as the preferred tree that which requires the fewest character state changes to explain the character-state distribution in the

data (Carmin & Sokal, 1965; Fitch, 1971; Fitch & Farris, 1974), that is to say, the tree that requires the fewest evolutionary steps to explain the observed traits of the taxa under consideration. Evolutionary change is measured differently in different forms of parsimony, each of which applies specific constraints on permissible character-state changes (Fitch & Margoliash, 1967; Farris, 1970; Swofford & Olsen, 1990), but the underlying minimisation of tree length (evolutionary change) constitutes the optimality criterion for all forms.

Generalized parsimony is an assimilated form of parsimony in which the conditions under which sequences evolve can be user-defined (Sankoff & Cedergren, 1983). Two types of algorithms are available: the exact algorithms (eg. Hendy & Penny, 1982; Swofford & Olsen, 1990) and the heuristic algorithms (eg. Farris, 1970). Exact methods identify all most-parsimonious trees but are computationally intensive, and only a limited number of sequences (usually <20) can be manipulated in one analysis (Hendy & Penny, 1982). Larger data sets require an heuristic approach, which does not guarantee the truly most-parsimonious tree.

A5.1.2.1 *inconsistency*

Under certain circumstances, parsimony analysis is subject to problems of inconsistency - the tendency of an estimated parameter to converge on the wrong value of that parameter as the sample size becomes infinite (Felsenstein, 1978). Substitutional saturation, heterogeneity in rates of change across sites, and base compositional bias can all contribute to the inconsistency of a phylogenetic estimation (Schmidt *et al.*, 1998). The evolutionary model under which parsimony becomes inconsistent was initially identified as one where the difference in evolutionary rates between some branches was large (Felsenstein, 1978). Subsequently, the juxtaposition of long and short branches was recognized as the source of inconsistency and the phenomenon referred to as "long branch attraction" (Hendy & Penny, 1989). The grouping together of long branches is more likely to produce minimal length trees, so incorrect topologies are chosen in which long branches are artefactually clustered (Swofford *et al.*, 1996). To lessen the possibility of long branch attraction, characters may be sampled to include only those that are phylogenetically meaningful. This achieves a reduction in homoplasy. Alternatively, or additionally, sequences may

be added into the analysis which are intermediate between distant sequences and therefore break up long branches. The choice of outgroups also affects branch length and judicious choice of outgroups may alleviate a tendency to inconsistency (Smith, 1994). The juxtaposition of long and short branches, and hence long branch attraction, can also be mitigated by homogeneous and representative sampling of the variation within the whole group (Milinkovitch *et al.,* 1996), so no branch is remarkably longer than another. However, this is an idealized option, often unavailable in real world data sets.

A5.1.3 Maximum Likelihood

The maximum likelihood method of phylogenetic inference computes the probability of the data set given the phylogeny, and then searches for the phylogeny that maximizes this probability (Edwards & Cavalli-Sforza, 1964; Felsenstein, 1981). The optimality criterion, in this case, is also based on a model of evolutionary change. The assumptions of the maximum likelihood model used in the PHYLIP package are :

1. Each site in the sequence evolves independently.
2. Each lineage evolves independently
3. Each site undergoes substitution at an expected rate that is defined by the user, together with its probability of occurrence (Felsenstein, 1981; Sidow *et al.*, 1992). The DNAML 3.5 program of the PHYLIP package incorporates this component to allow for site rate heterogeneity because the assumption of equal rates of substitution across sites is not only unrealistic (especially in protein-coding genes where codon degeneracy makes third codon positions hypervariable) but when contravened, results in strongly biased trees (Gaut & Lewis, 1995).
4. All sites are included in the estimation of the phylogeny. This differs from parsimony analysis in which only "phylogenetically informative" sites are included in the calculation of trees. Omitting uninformative sites in ML computation would bias branch lengths by making those with uninformative sites relatively too long (Felsenstein, 1995), though ML is robust to the exclusion of invariant sites (Penny *et al.*, 1996).

ML is a method that displays both consistency and efficiency (Felsenstein, 1973, 1982b). Statistical consistency is the certainty that a method will converge on the correct estimate when unlimited data are available. Efficiency ensures that the lowest possible variance is achieved of the estimate around the true value, with unlimited data (Felsenstein, 1983). Because inconsistency is a factor that can bedevil parsimony analysis, the availability of an alternative phylogenetic technique that is free of the problem makes it possible to verify relationships defined by parsimony using ML. Maximum likelihood appears to be less sensitive to violations of its basic premises than are other methods (Huelsenbeck, 1995b). Furthermore, ML (and distance) methods are less sensitive to contravention of the assumption of low rates of change than is parsimony (Felsenstein, 1983).

The application of ML to protein sequences involves the choice of an appropriate model of amino acid substitution. As in parsimony analyses, a version of the Dayhoff transition probability matrix is used here.

A5.2 Assessing Phylogenetic Signal

Before any inference is made of a data set, it is prudent to establish the presence of non-random structure in that data set. Data which contain no character covariation over that expected from random sampling, display a relatively symmetrical tree-length frequency distribution (Fitch, 1979, 1984). As character covariance increases, the tree-length frequency distribution becomes increasingly left-skewed (Hillis & Huelsenbeck, 1992). The g_1 statistic is a measure of this skewedness and therefore represents a measure of phylogenetic signal for that data set (Hillis, 1991). There is a strong correlation between the g_1 statistic and the capacity of parsimony to establish a true phylogeny. The statistic is particularly sensitive to the number of taxa and less so to the number of characters used in the analysis. For four state character data, data sets which generate a g_1 statistic less (more negative) than the values shown in Table 5.2 are significantly more structured than a random data set at a confidence level of P=0.01.

	10 taxa	15 taxa	25 taxa
10 characters	-0.42	-0.25	-0.18
50 characters	-0.36	-0.20	-0.13
100 characters	-0.33	-0.19	-0.12

Table 5.2 - Values of the g_1 statistic for different numbers of taxa and characters, below which the dataset can be considered to contain significant character covariance, or non-random structure (taken from Hillis & Huelsenbeck, 1992).

A5.3 Consensus trees

With maximum parsimony and ML inference, more than one tree may fulfil the optimality criterion. Multiple most-parsimonious or most-likely trees do not constitute a logical predicament, and often represent a highly accurate measurement (Penny & Hendy, 1986). However, an integration of multiple solutions into a single tree often facilitates easier interpretation. Different degrees of consensus may be derived using different sets of constraints. "Strict" consensus trees represent only those groupings that occur in all of the resultant trees (Sokal & Rohlf, 1981). "Majority rule" consensus trees represent those groupings that occur in a majority of the possible trees (Margush & McMorris, 1981); this "majority" may be user specified eg. a 50% majority tree will show those monophyletic groupings that occur on at least 50% of the trees. The phylogenetic package PHYLIP implements a modified 50% majority rule consensus program. All clades that occur in >50% of trees appear on the consensus tree, together with clades occurring with less frequency as long as these groups do not contradict more frequently occurrent clades already established on the consensus tree (Felsenstein, 1995).

A5.4 Bootstrapping

When a method is sensitive to finite sampling, the estimate of a population parameter established from a subset of that population will be incorrect. This constitutes random error. In phylogenetic analysis, several methods are used to counteract the error that arises from finite data sets, the most common of which is the non-parametric bootstrap (Felsenstein, 1985). The

principle upon which bootstrapping is based is that, in the absence of infinite data, further data sets are generated from the original data set by reiteratively and randomly resampling it, with replacement (Efron, 1979). When the size of the original data set is large, character states should be represented in the same proportion as occurs in the underlying population, and bootstrap resampling is equivalent to sampling from that population (Efron, 1982). Even if the data set is small, the resampling is a good approximation to sampling from the population (Felsenstein, 1985). For each new data set created in this way, the phylogeny is calculated and the proportion of data sets that supports a particular branching is indicative of the internal support for each clade of the tree. This is the bootstrap confidence limit, P (Felsenstein, 1985). Conventionally, a bootstrap value, or bootstrap proportion, of P=70 has been accepted as providing adequate support for a clade (Zharkikh & Li, 1992; Hillis & Bull, 1993). Hillis & Bull (1993) propose that under most circumstances, P=70 is equivalent to a 95% probability that the corresponding clade is real. The bootstrap P value associated with a certain node only reflects a particular data set and inference method. A method that is inconsistent could produce an incorrect tree with statistically significant bootstrap support (Felsenstein, 1985). The trees generated from bootstrap resampling are integrated into a single "consensus" tree as described above.

A5.5 Character Weighting

Assigning less weight to characters known to contribute less to meaningful phylogenetic signal is used frequently to reduce phylogenetic "noise" in a data set (Irwin *et al.*, 1991; Simon *et al.*, 1994). Weighting is usually related to the rate of evolution: slowly evolving characters are weighted more highly as their probability of change is lower and the significance of their change therefore more meaningful (Felsenstein, 1981). Also, rapidly evolving characters are more likely to be saturated with superimposed changes, and therefore contribute more homoplasy to an analysis than will more slowly evolving characters (Wheeler, 1986). This is especially true with distantly related sequences. Weighting can also be used to minimize the effect on an analysis of characters known to violate the assumptions that underpin a method of inference (Swofford *et al.*, 1996). Ideally, a weighting scheme should have a justifiable

rationale based on critical examination of the data (Milinkovitch *et al.*, 1996). Weighting may be *a priori*, predicated on assumptions made about the data from the outset (Carpenter, 1994; Allard & Carpenter, 1994), or *a posteriori*, based on the contribution the characters themselves make to the resulting phylogeny (Farris, 1969; Neff, 1986). The issue of character weighting is polemical and many different weighting schemes are employed.

A5.5.1 unweighted

Opinions conflict regarding the option to differentially weight sequence character transitions. It is often proposed that no weighting should be applied without some external justification (Allard & Carpenter, 1994). On the other hand, external justification is repudiated by others as removing systematic analysis from its empirical base (Forey *et al.*, 1993), an argument against any form of differential character weighting. However, an assumption of equal character weights is a vigorous assumption in itself (Swofford *et al.*, 1996) and it would seem equal weighting requires as much justification as any other method. One instance where equal weighting is justified is at very low rates of character change. In this circumstance, differences in rates of change between characters have little effect on parsimony analysis. Slowly evolving characters should be weighted equally. Only when rate disparities become extreme is differential character weighting recommended (Felsenstein, 1981, 1982a, 1983). Another situation in which the need for differential character weights is obviated occurs when a data set represents the variability of a group of sequences both homogeneously and extensively. Character weighting will contribute nothing further to the outcome (Milinkovitch *et al.*, 1996).

A5.5.2 transversion weighting

It has been shown that transitional changes occur more frequently in evolution than transversional changes (Brown *et al.*, 1982) and that transitional changes may become saturated even over small genetic distances (Swofford *et al.*, 1996). Consequently, it is common practice to weight transversional changes more heavily than transitional changes and a transition:transversion ratio (ts:tv) of between 2 and 100 is commonly used. Transitional changes may be deleted

from the computation altogether where transitions are presumed to be absolutely saturated. In protein-coding genes, third codon position transitions are expected to become saturated much more quickly than those at other positions and this can be factored into an analysis. Both transition parsimony and weighted parsimony are extremely sensitive to the presumed ts:tv bias (Huelsenbeck & Hillis, 1993) and therefore care must be exercised in the choice of weighting. In practice, however, a wide range of ts:tv weightings often produces the same outcome (Hillis *et al.*, 1994).

A5.6 Outgroup Choice

The outgroup in a phylogenetic reconstruction defines the monophyly of the ingroup and determines the rooting of the tree (Swofford *et al.*, 1996). In so doing, it also establishes character polarisation (Farris, 1972) and to do this accurately, the outgroup must be relatively closely related to the ingroup because homoplastic similarity will increase with distance and noise will be introduced into the phylogenetic signal (Maddison *et al.*, 1984; Milinkovitch, 1995). Multiple outgroups, ideally all closely related to the ingroup, are recommended in maximum parsimony inference to amplify character polarisation (Milinkovitch *et al.*, 1996; Nixon & Carpenter, 1993) Because outgroups often have long branches (Hendy & Penny, 1989; Smith, 1994), the use of multiple outgroups may also reduce the likelihood of long branch attraction in parsimony analysis. Species sensitivity is a problem with some data sets, and the sampling of successive outgroups can give different topologies. Therefore, it is advisable to sample a range of outgroups if possible (Lecointre *et al.*, 1993); high congruence amongst trees is an index of correct rooting (Milinkovitch *et al.*, 1996). A single outgroup, however, is recommended for distance analyses (Swofford *et al.*, 1996). Choice of outgroup is especially important for small data sets because they are more vulnerable to the impact of character mispolarisation than are large sets (Ritchie *et al.*, 1997).

A5.7 Choice of Taxa

A5.7.1 representation

Because a sequence may not be truly representive of a taxon, two or more representative sequences per presumed monophyletic group are recommended. Inadequate sampling of monophyletic groups will impact especially on the reliability of bootstrap values (Lecointre *et al.,* 1993; Kim, 1996).

A5.7.2 species sensitivity

Both systematic and random error can distort a tree so that the inferred branching order is dependent on the taxa included. There are ways of identifying taxa which exert an anomalous effect on tree topology, including aberrant homoplasy indices in parsimony analysis or likelihood ratio statistics in ML, the deviations of which can be demonstrated in simulation studies (Swofford *et al.*, 1996). Some sequences seem to have specific idiosyncratic and reproducible effects on tree topology, usually attributable to complex interactions between several sequences (Lê *et al.,* 1993). These effects may be diluted out with the addition of further sequences. Larger data sets are, therefore, more independent of the effects of species sampling than are small data sets (Lecointre *et al.*, 1993).

A5.7.3 redundant taxa

Redundant taxa are those sequences that may be removed from a phylogenetic analysis to render the variability depicted in the data set more representative of the underlying population of sequences. They basically embody an over-representation of members of some monophyletic groups. Redundant sequences are identified using pairwise uncorrected distances to distinguish the most closely related pairs of sequences in a data set. One of this pair is deemed redundant and it is removed from the matrix together with all pairwise distances between it and other sequences. This process is repeated until further repetition would result in the removal of the last member of some monophyletic group (Milinkovitch *et al.,* 1996). In this way, the data set is reduced to informative

taxa only. Removing redundant taxa can reduce a data set to computational manageability while retaining representational veracity. Furthermore, the phylogenetic "noise" introduced by redundant sequences can bias an outcome considerably. It has been shown that the removal of redundant taxa can reduce the mispolarization of characters that results from homoplasy, and make estimates of phylogeny less sensitive to outgroup choice (Milinkovitch *et al.*, 1996).

A5.8 Other Factors that Affect Phylogenetic Inference

A5.8.1 base composition

Heterogeneity of base composition between different species in an analysis can affect the outcome of a phylogenetic analysis (Rzhetsky & Nei, 1995), especially if the bias does not follow phylogenetic trends (eg. Pettigrew, 1994). Anomalous branch attraction has been shown to result from shared base composition (Flook & Rowell, 1997). Analysis of highly conserved protein-coding genes seems less susceptible to distortion by base compositional bias than other genes, such as rRNA genes (eg. Roger *et al.*, 1996). Artefacts arising from base compositional heterogeneity between sequences can be avoided by using the LogDet transformation in distance matrix methods of inference which corrects for G+C bias, or by investigation of protein sequences rather than nucleic acid sequences (Loomis & Smith, 1990; Hasegawa *et al.*, 1993; Leipe *et al.*, 1993).

A5.8.2 sequence length

Sequence length has been cited as the most important determinant in the recovery of a reliable phylogeny (Hillis *et al.*, 1994; Flook & Rowell, 1997). Increase in the number of sampled characters can be accomplished in species phylogenies by extension of the gene regions compared or the simultaneous or combined analysis of additional genes. A gene phylogeny, however, is limited to the sequence length of the gene and if the gene is short, as are the metallothioneins, the consequences of this for the analysis must be considered.

A5.8.3 codon usage

Differences in codon usage occur between species and between genes (Bailey & Freshwater, 1997). It has been suggested that codon bias is related to the expression level of the gene (Shields *et al.*, 1988), and may therefore differ between members of a multigene family within the same organism. Biased codon usage will affect the substitution probabilities within sequences (Fitch & Markowitz, 1970) and therefore the phylogenies based on these sequences. In these cases, protein sequence phylogenies can be used to confirm nucleic acid sequence phylogenies.

A5.9 Amino Acid vs. DNA Analysis

Some phylogenetic information is lost in the translation of nucleic acids to protein sequences. An analysis of nucleotide sequences is expected to be more informative, especially for closely related species, than a protein sequence analysis as synonymous amino acid substitutions can also be addressed. However, protein sequences are often used in an analysis to remove saturated characters (often the third site position of codons) which detract from phylogenetic signal for more distantly related sequences. Furthermore, the base composition, especially at the third codon position, varies between species and can therefore differ between lineages, perturbing the data.

A further limitation to the use of DNA arises because assumption of equal rates of change at sites is violated due to the degeneracy of the genetic code (Reeves, 1992). This is easily corrected by allowing relative rates to be specified on a site by site basis (Felsenstein, 1995). However, amino acid sequences are often used when ML trees especially are calculated from protein-coding genes, to avoid violation of this assumption. A further, more cogent reason for using protein sequences in phylogenetic analysis is that some sequences may have been retrieved only as amino acid sequences and not at the nucleic acid level (eg. pigeon MT1 and MT2). It is important to recognize that an amino acid data set is much less variable than its corresponding nucleic acid set, and resolution may be lost in more recent nodes of the tree.

A5.10 Reading phylogenetic trees

Phylogenetic reconstructions are presented in standardised tree format. Relationships are most easily understood if trees are treated as a succession of bifurcating branches or "clades". The two members of any bifurcation are more closely related to one another than either is to any other taxon outside that clade. For example:

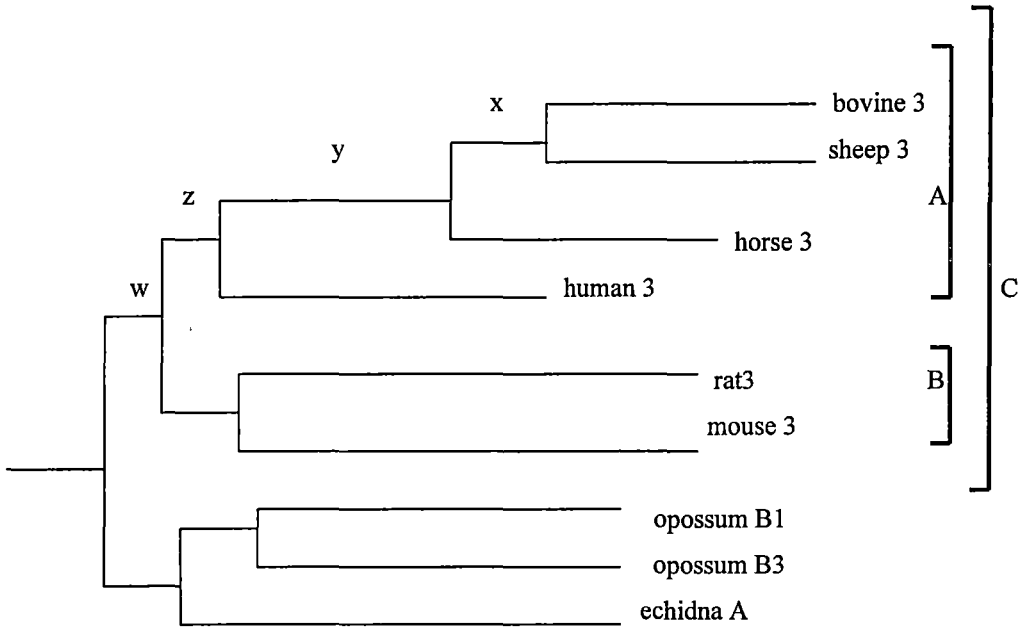


Figure 5. 19 An hypothetical tree to illustrate the relationships within a phylogeny and the concept of clades, as described in the text.

In this tree, bovine3 and sheep3 represent a pair of most closely related sequences, and together represent a single clade or monophyletic grouping ie. a bifurcation at the endpoint of branch x. Of the remaining taxa on the tree, horse3 is most closely related to this bovine/sheep clade and these three taxa themselves form another monophyletic grouping, or bifurcation of the branch y. By the same process, the human3 sequence is next most closely related and can be incorporated into a larger, 4-taxon clade (which we may call for convenience clade A), resulting from the bifurcation of branch z. The members of clade A are less closely related to one another than are the members of the smaller, initial bovine/sheep clade. Rat3 and mouse3 are closely related and form an independent monophyly (clade B). The relationship between the clades A and B

is more distant than are the relationships within either clade. However, clades A and B themselves form clade C, the bifurcation of branch w, and taxa within clade C are more closely related to one another than any of them are to taxa outside the clade A/clade B bifurcation - that is, to the echidna and opossum sequences. In this manner, the relationships within a tree can be deduced.

A5.11 Definition of terms

ANGIS - the Australian National Genome Inforation Service, a data base manipulation facility sited at the University of Sydney, Australia.

bootstrap - the non-parametric bootstrap is a technique used to assess the reliability of a phylogenetic grouping on a tree. It is based on resampling from the original data set and is discussed in section A5.4.

bootstrap proportion (bootstrap confidence limit, bootstrap value) - is the proportion of resampled data sets that supports a particular grouping on a tree, expressed as a percentage. It is discussed in section A5.4.

branch - a branch connects two nodes. Branch length represents expected number of substitutions per site along that branch, which may correspond to either evolutionary time, substitution rate or both.

branch-and-bound - an algorithm of parsimony analysis in which the most parsimonious tree or trees are guaranteed retrievable (cf. heuristic).

character state - each character within a sequence has several possible options (eg. A, T, C, G for DNA sequences). The character state is that option adopted by a character of a particular sequence. Hence: character state change; character state weight etc.

clade - a monophyletic grouping (see below)

cluster - to form a monophyletic grouping

cluster analysis - methods by which a collection of n isolated taxa are progressively amalgamated into larger clusters on the basis of common properties, until only a single cluster containing n taxa remains.

consensus tree - is that tree produced from the integration of multiple solutions (trees) into a single tree. The concept and construction of the consensus tree is discussed in section A5.3.

g_1 statistic - represents a measure of character covariance or structure in a data set beyond that attributable to random signal. Its use is described in section A5.2.

genetic distances - is a measure of dissimilarity between sequences. Uncorrected genetic distance is equivalent to the proportion of sites at which two sequences differ. Genetic distances may be "corrected" to compensate for the sites at which multiple substitutions have occurred over time, which would otherwise cause an underestimation of genetic distance. The transformations that are used to correct genetic distance measures are discussed in section A5.1.1.

heuristic - an algorithm used in parsimony analysis in which the most-parsimonious tree is not guaranteed, and may not be derived. Various indices are evaluated to determine how close the phylogeny constructed in this way may be to the "true" tree.

homology - character likeness that arises from shared ancestry.

homoplasy - character likeness that arise from causes other than shared ancestry.

inconsistency - see section A5.1.2.1.

informative site - an informative character site in an alignment of sequences is one which displays more than one character state, and these different states contribute to an understanding of the inter-relationship of the sequences of the data set. An uninformative site is one in which character states at that site do not contribute meaningfully to a phylogenetic reconstruction (usually because too few states are present).

lineage - those taxa forming a direct line of descent; a continuous line of antecedence.

log likelihood value - in ML, the likelihood that a particular tree will have produced the given data set is calculated and the tree with the highest likelihood score is deemed the "best" tree. Likelihood values are given as logarithms, ie. log likelihood values.

long branch attraction - is the term used for the phenomenon whereby branches of significantly dissimilar length on a tree can produce an incorrect grouping of taxa, for mathematical reasons, and which is enhanced by the addition of further taxa. This phenomenon is particularly relevant to parsimony analysis and is discussed under "inconsistency" in section A5.1.2.1.

molecular clock - the presumption that sequence substitutions occur at an approximately equivalent rate across character sites, genes and lineages.

monophyletic - a monophyletic grouping is the product of a single branch. Monophyletic groupings, or "clades" are illustrated in Fig. 5.19 (above).

node - branch points, or points of bifurcation, within a phylogenetic tree are the "internal nodes", and correspond to ancestral taxa or sequences. Contemporary taxa/sequences correspond to terminal nodes, or "tips" of the branches on the tree.

orthologue - genetic sequences related by direct descent; homologous sequences reflecting the descent of species; sequences that share a direct common ancestry eg. mouse β-globin and human β-globin.

orthology - see orthologue

outgroup - the taxon or taxa chosen such that the remaining taxa form a monophyly (the ingroup). The role and implications of outgroup choice are discussed in section A5.6.

paralogue - sequences that are related by a duplication event; can co-exist in the same organism eg. mouse α-globin and mouse β-globin.

paralogy - see paralogue

PAUP - "phylogenetic analysis using parsimony": a parsimony-based analysis package written by D. L. Swofford

PHYLIP - "phylogeny inference package": an analysis package utilising distance, parsimony and maximum likelihood methods, written by J. Felsenstein.

redundant taxa - are those taxa that over-represent members of a monophyletic group in a data set. Their significance to phylogenetic reconstruction is discussed in section A5.7.3.

saturation - multiple substitution at a particular site that has occurred so often that it cannot be corrected for computationally. Saturation leads to underestimation of genetic distance.

sister group - member of a monophyly

spectral analysis - a method of character transformation that evades problems of inconsistency

stem reptile - the reptile antecedent of both the mammals and the birds.

transition - a transitional change is the substitution at a sequence site of a purine for a purine, or a pyrimidne for a pyrimidine.

transversion - a transversional change is the substitution of a pyrimidine for a purine, or a purine for a pyrimidine.

ts:tv - transversional substitutions occur less frequently than transitional ones, and therefore have greater phylogenetic significance. The transition:transversion ratio specifies numerically the degree of importance given to a transversional change over a transitional one in calculation of a phylogeny, as described in section A5.5.2.trees.

WebANGIS - the Australian National Genome Inforation Service available through the internet.

CHAPTER 6 : CONCLUDING REMARKS

6.1 Characterisation of the marsupial metallothioneins

Class I metallothioneins have been the object of extensive study for the last 40 years. However, no metallothionein has been characterised till now from a marsupial or a monotreme species. Five MT cDNA sequences are presented here from the opossum *Monodelphis domestica* and two from the echidna *Tachyglossus aculeatus*. Phylogenetic analysis of the vertebrate MT gene families predicts further MT genes in these species, specifically an MT3 homologue, though the occurrence of other MT3-derivative genes cannot be excluded. Sequence characteristics of these MT cDNAs and characteristics of their cognate proteins suggest that the marsupial and monotreme metallothioneins cannot be easily correlated with those of eutherian mammals.

6.2 Expression

The expression of MT transcript in the opossum is basically similar to the patterns reported for eutherian MT1 and MT2. Parallels can also be drawn between the expression of eutherian MT3 in the dentate gyrus of the hippocampal region of the brain and the signal observed in the opossum brain with *in situ* hybridisation, using a probe to transcripts of known isoforms. Further work to confirm these findings and to investigate any functional correlation between opossum MTs and eutherian MT3 may therefore prove important.

Extremely high levels of metallothionein mRNA are here reported in the opossum tongue, by northern blotting and *in situ* hybridisation. These transcripts are also highly localised to particular cell types. No previous report has been made of the relative levels of MT in the mammalian tongue. If this phenomenon is widespread in the mammals, its significance is enhanced by findings that the tongue is one of the two tissues in which the complete metallothionein locus is known to be expressed (Moffatt &Seguin, 1998). The tongue not only expresses the whole MT locus, but does so at very high levels. There may be further significance in the fact that the tongue is one of the few sites of expression of MT4, possibly the most ancient of the mammalian metallothioneins.

Very high levels of transcript are also demonstrated here in the opossum salivary gland. Relative levels of MT in mammalian salivary gland have not been previously investigated. Whether these levels are consistently high throughout all mammals, or correlate with heavily salivating species such as the opossum, has yet to be established. No MT mRNA was demonstrated in the myoepithelial cells of the salivary gland, despite the prominence of this cell type in the opossum. This supports the contention of Shrestha *et al.* (1996) that MT does not occur in this cell type.

6.3 Developmental expression

Expression of MT in the neonatal liver is essentially different from that in the eutherian neonate. This may be attributable to the different utilisation of oxygen concomitant with the transient ectothermy of the marsupial young, and the activity of MT as a moderator of oxygen-mediated damage. It may also, or otherwise, correlate with gradual development of liver processes in the marsupial, (including the management of metals), which contrasts markedly with the sudden recruitment of the eutherian liver into full-blown activity in the relatively mature neonate. Further investigation of these differences may provide insights into the role of MT in the developing liver and account for the well-documented peak of hepatic expression in the perinatal eutherian.

6.4 Phylogeny

Examination of the marsupial and monotreme MT sequences has clarified the evolution of the mammalian MT isoforms in several ways.

Firstly it has demonstrated a genetic basis to the distinction of the various eutherian isoforms of MT. MT1 sequences cluster with other MT1 sequences (with allowance made for homogenisation due to gene conversion); MT2 sequences with other MT2 sequences; and MT3 with MT3. These groupings conform with certain characters of the cognate proteins, including charge separability, by which the proteins were first classified.

Furthermore, by reference to the marsupial and monotreme MT sequences it is possible to date the multiplication of the eutherian locus, based on

extrinsic, palaeontological criteria. The eutherian locus evolved from one or two ancestral genes after the marsupial-eutherian split and before the segregation of the rodents, approximately 120 million years ago. At this point, the basic four-member gene family arose in the eutherian mammals. Reference to the marsupial sequences also provides a markedly more precise estimate than that given for the duplication of *MT2* of 45-120 million years ago, derived directly from sequence data by Griffith *et al.* (1983). The expansion of the marsupial and monotreme MT gene families, from an MT3-like ancestral sequence, also occurred from this time. By the same process, the date or dates of the further multiplication of various metallothionein 1 or 2 genes in individual eutherian groups can be estimated from the divergence dates of those groups.

The evolution of the metallothioneins provides an important framework for their classification. It is clear from these data that the definition of isoforms currently applied to the eutherian metallothioneins cannot be extended to other groups. Eutherian MT1 and MT2 have no counterpart even in the closely related marsupials. Any revision of classification and nomenclature must accommodate these insights.

From a more general perspective, this analysis of the mammalian metallothioneins supports the Marsupionta hypothesis of mammalian evolution, in keeping with the recent analysis of complete mitochondrial genomes by Jancke *et al.* (1997). Under this hypothesis, marsupials and monotreme derive from a common ancestor which separated from the eutherian lineage some 130 million years ago.

A new mechanism of gene family evolution, implicating environmental factors, is proposed in a testable hypothesis in Chapter 5, and the ramifications briefly discussed. The metallothioneins have been used as model genes in many instructive and innovative experimental situations, and it is therefore apposite that the metallothionein gene family could contribute to the understanding of the mechanisms of gene family evolution.

6.5 MT3

No cDNA corresponding to an *MT3* was isolated from the opossum cDNA libraries. Phylogenetic analysis of the vertebrate *MT* sequences, however,

predicts the occurrence of an MT3 isoform in the marsupials and the monotremes. From this progenitor *MT3* arose the five opossum metallothioneins described in this thesis. Similarly, the eutherian MT1 and MT2 isoforms are coded by genes that arose from sequential duplications of either an MT4 (hypothesis 1) or an MT3 (hypothesis 2) ancestral gene. Interestingly, this implies that the whole-body expression that characterises the metallothioneins of the fish, amphibia and birds, originally pertained to the MT3 (or MT4) isoform in the ancestor common to all the mammals. After the expansion of the MT locus to four genes in the eutherians, the expression of the ancestral genes was reduced to relic sites in the brain and limited regions of squamous epithelia. If the MT3 isoform serves a different function in the eutherian brain than the MT1 and MT2 isoforms (as is mooted), a functional switch may also be proposed. If MT4 is orthologous to pigeon MT1, and therefore the ancestral form, its occurrence in the pigeon liver corroborates a former more widespread distribution of MT4 in the vertebrate body - with localisation of distribution occurring subsequently in the mammals. Discovery of *MT4* in the marsupials would clarify the phylogenetic position of *MT4* as the ancestral mammalian gene, and work is currently underway to this end.

6.6 Opossums and MT research

The opossum is becoming increasingly useful and utilised as a model for various biological systems (eg. Tyndale-Biscoe & P. A. Janssens , 1988; Robinson & Dooley, 1995; Kusewitt & Ley, 1996). There are instances in which, due to certain features peculiar to marsupials, they are also potentially excellent models for MT research. The existence of the photoreactivating enzyme, DNA photolyase, in the opossum reduces lethal, mutagenic and carcinogenic effects of ultraviolet light by catalyzing near UV or visible light-dependent repair of cyclobutane pyrimidine dimers in DNA (Kato *et al.*, 1994). This enzyme does not occur in eutherian mammals. MT has been implicated in DNA repair (Blattner *et al.*, 1998; Robson *et al.*, 1994). Metallothionein, furthermore, is elevated and may be protective in UV-irradiated skin (Hansen *et al.*, 1997), and the MT-null mouse provides direct evidence of the photoprotective effect of cellular MT in the skin (Hanada *et al.*, 1998). A

comparison of the eutherian and marsupial systems, and the expression of MT in the presence and absence of photolyase, may therefore be insightful. MT is also implicated in the progression of malignant melanoma (Zelger *et al.*, 1993) and the opossum is proving a useful model of melanoma research (Robinson & Dooley, 1995; Kusewitt & Ley, 1996).

6.6.1 Spinal chord regeneration

The opossum *Monodelphis domestica* has become, over the last few years, the focus of intensive research on spinal chord regeneration. In the immature mammalian spinal chord, spontaneous regeneration is possible after injury (Saunders *et al.*, 1995; Varga *et al.*, 1995a, 1995b). After a critical developmental stage, this plasticity is lost, coincident with the onset of myelination and the activity of neurite inhibitory proteins. The facility with which the opossum central nervous system (CNS) can be maintained, and its development sustained, *in vitro* (Nicholls *et al.*, 1990) provides an excellent model for the study of spinal chord regeneration. In the opossum, the critical period for regenerative processes ends at P12 (Treherne *et al.*, 1992). Evidence exists to suggests that other neurite inhibitory molecules, additional to the myelin-asociated factors, are expressed in the CNS increasingly from P12 (Varga *et al.*, 1995b; Maclaren, 1996). This coincides with the rise in cerebral metallothionein expression in the opossum brain described in Chapter 4. Reactive astrocytes have been shown to express factors that are inhibitory to axonal growth after CNS injury (Bovolenta *et al.*, 1993), and this inhibitory activity increases as development proceeds (Smith *et al.*, 1986; Ajemian *et al.*, 1994). The demonstrated *in vitro* growth inhibtory activity of MT3 (Uchida *et al.*, 1991), its localisation to reactive astrocytes and its increasing levels from birth to maturity (Masters *et al.*, 1994a) make MT3 a candidate for this inhibitory protein. Isolation of opossum MT3 and its application in the assay system provided by the cultured CNS of the opossum may contribute to our understanding of the physiological significance of the growth inhibitory function attributed to mammalian MT3.

In summary, the opossum *Monodelphis domestica* lends itself in several ways to the profitable extension of MT research. Characterisation of the opossum MT gene family is a first step in this application.

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Appendix I - Complete MT DNA Data Set

49 taxa 207 characters
Gaps introduced into sequences to permit alignment are represented ".".

Genbank Accession		1 50
U58774	bream	ATGGACCCT. .....TGCGA GTGCTCTAAG ACTGGAACCT GCAACTGCGG
X06749	chicken	ATGGACCCTC AGGACTGCAC TTGTGCTGCT GGTGACTCCT GCTCCTGTGC
U08105	cod	ATGGATCCC. .....TGCGA CTGCGCCAAG ACTGGAACCT GCAACTGCGG
U34231	duck	ATGGACCCCC AGGACTGCAC ATGTGCTGCT GGTGACTCCT GCTCCTGTGC
	echidnaA	ATGGACCCTC AGGACTGCGG CTGTGCCACT GGTGGCTCTT GTGCCTGTGC
	echidnaB	ATGGACCCTC AGAACTGCGG CTGTGCCACT GGTGGCTCTT GTACCTGTGC
X97271	goldfish	ATGGATCCC. .....TGCGA TTGCGCCAAG ACTGGAGCTT GCAACTGTGG
J00061	hamster1	ATGGACCCC. ..AACTGCTC CTGCTCCACC GGCAGCACCT GCACCTGCTC
J00062	hamster2	ATGGACCCC. ..AACTGCTC CTGTGCTACA GATGGATCCT GCTCCTGCGC
K01383	human1a	ATGGACCCC. ..AACTGCTC CTGCGCCACT GGTGGCTCCT GCACCTGCAC
M13484	human1b	ATGGATCCC. ..AACTGCTC CTGCACCACA GGTGGCTCCT GTGCCTGCGC
M10942	human1e	ATGGACCCC. ..AACTGCTC TTGCGCCACT GGTGGCTCCT GCACGTGCGC
M13003	human1f	ATGGACCCC. ..AACTGCTC CTGCGCCGCT GGTGTCTCCT GCACCTGCGC
J03910	human1g	ATGGACCCC. ..AACTGCTC CTGTGCCGCT GGTGTCTCCT GCACCTGCGC
X64834	human1h	ATGGACCCC. ..AACTGCTC CTGCGAGGCT GGTGGCTCCT GCGCCTGCGC
X97261	human1r	ATGGACCCC. ..AACTGCTC GTGCGCCACT GGGGGCTCCT GCTCCTGTGC
X65607	human1x	ATGGACCCC. ..AACTGCTC CTGCTCGCCT GTTGGCTCCT GTGCCTGTGC
J00271	human2	ATGGATCCC. ..AACTGCTC CTGCGCCGCC GGTGACTCCT GCACCTGCGC
D13365	human3	ATGGACCCTG AGACCTGCCC CTGCCCTTCT GGTGGCTCCT GCACCTGCGC
U07807	human4	ATGGACCCCA GGGAATGTGT CTGCATGTCT GGAGGAATCT GCATGTGTGG
Z72483	icefishA	ATGGATCCC. .....TGCGA GTGCACCAAA AGTGGGACCT GCAACTGCGG
Z72484	icefishB	ATGGATCCC. .....TGTGA GTGCTCCAAA AGTGGGACCT GCAACTGCGG
AJOO7951	icefishC	ATGGATCCC. .....TGCGA GTGCTCCAAA AGTGGGACCT GCAACTGCGG
X59393	loach	ATGGATCCT. .....TGTGA CTGCTCAAAA ACTGGAACAT GCAACTGCGG
V01533	monkeya	ATGGACCCC. ..AACTGCTC CTGCGCCACT GGTGTCTCCT GCACCTGCGC
V01532	monkeyb	ATGGATCCC. ..AACTGCTC TTGCGTCGCC GGTGACTCCT GCACCTGCGC
J00605	mouse1	ATGGACCCC. ..AACTGCTC CTGCTCCACC GGCGGCTCCT GCACTTGCAC

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K02236      mouse2       ATGGACCCC. ..AACTGCTC CTGTGCCTCC GATGGATCCT GCTCCTGCGC
S72046      mouse3       ATGGACCCTG AGACCTGCCC CTGTCCTACT GGTGGTTCCT GCACCTGCTC
U07808      mouse4       ATGGACCCTG GGGAATGCAC GTGTATGTCT GGAGGGATCT GCATCTGCGG
U34230      muscovy      ATGGACCCCC AGGACTGCAC ATGTGCTGCT GGTGACTCCT GCTCCTGCGC
            opossumB3    ATGGACCCC. ..AACTGTGA CTGCGTGAGC GGAAGTTCTT GCACCTGTGC
            opossumB1    ATGGACCCC. ..AAATGCAA CTGCGAGAGT GGAGGTTCTT GCACCTGTGC
            opossumL2    ATGGACCCC. ..GACTGTGA CTGCGTGAGC GGTGGCTCTT GCACCTGTGC
            opossumL3    ATGGACCCC. ..AACTGTAA CTGCGCCAAC GGTGGCTCCT GCACCTGCGG
            opossumx5    ATGGACCCC. ..AATTGTGA CTGTGGGAAT GGTGCCTCTT GTACCTGTGC
X97272      perch        ATGGATCCC. .....TGCGA GTGCTCCAAG GGTGGAACCT GCAACTGCGG
M29515      pig          ATGGACCCC. ..AACTGCTC CTGCCCCACA GGCGGCTCCT GCAGCTGCGC
X59392      pike         ATGGATCCT. .....TGTGA ATGCTCCAAA ACTGGATCTT GCAACTGTGG
X07790      rabbit1      ATGGACCCC. ..AACTGCTC CTGCGCCACA GGCAACTCCT GCACCTGTGC
M11792      rat1         ATGGACCCC. ..AACTGCTC CTGCTCCACC GGCGGCTCCT GCACCTGCTC
M11794      rat2         ATGGACCCC. ..AACTGCTC CTGTGCCACA GATGGATCCT GCTCCTGCGC
S65838      rat3         ATGGACCCTG AGACCTGCCC CTGTCCTACT GGTGGTTCCT GCACCTCCTC
AF008583    Ambystoma    ATGGAC.... .....TGCGC ATGCGCCACT GGCGGCTCCT GCTCTTGTGC
X04626      sheep1a      ATGGACCCG. ..AACTGCTC CTGCCCCACT GGCGGCTCCT GCAGCTGCGC
X07974      sheep1c      ATGGACCCC. ..AACTGCTC CTGCTCCACT GGCAGCTCCT GCAGCTGCGC
X07975      sheep2       ATGGATCCC. ..AACTGCTC CTGCACCGCG GGTGAATCCT GCACGTGCGC
M22487      troutb       ATGGATCCT. .....TGTGA ATGCTCTAAA ACTGGCTCTT GCAACTGCGG
M96729      Xenopus      ATGGACCCTC AGGACTGCAA ATGCGAAACA GGTGCTTCCT GCTCCTGTGG

                         51                                                100
bream       AGGATCCTGC ACATGCACAA ACTGCTCCTG CACCTCCTGC AAGAAGAGCT
chicken     TGGGTCGTGC AAGTGCAAGA ACTGCCGCTG CCGGAGCTGC CGCAAGAGCT
cod         CACATCATGC ACCTGCGCAA ACTGCTCCTG CACAAAATGC AAGAAAAGTT
duck        TGGGTCCTGC AAGTGCAAGA ACTGCCGCTG CCGGAGCTGC CGCAAGAGCT
echidnaA    GACATCCTGC AAATGCAAAG ACTGCCGGTG CACTTCGTGC AAGAAAAGCT
echidnaB    TGGATCCTGC AAGTGCAAAG ACTGCCGGTG CACTTCGTGC AAGAAAAGCT
goldfish    TGCCACCTGC AAGTGCACCA ATTGCCAGTG TACAACCTGC AAGAAGAGTT
hamster1    CAGTTCCTGT GGCTGCAAAG ACTGCAAGTG CACCTCCTGC AAGAAGAGCT
human1a    TGGCTCCTGC AAATGCAAAG AGTGCAAATG CAACTCCTGC AAGAAGAGCT
human1b    CGGCTCCTGC AAGTGCAAAG AGTGCAAATG TACCTCCTGC AAGAAGTGCT
human1e    CGGCTCCTGC AAGTGCAAAG AGTGCAAATG CACCTCCTGC AAGAAGAGCT
human1f    TGGTTCCTGC AAGTGCAAAG AGTGCAAATG CACCTCCTGC AAGAAGAGCT
human1g    CAGCTCCTGC AAGTGCAAAG AGTGCAAATG CACCTCCTGC AAGAAGAGCT
human1h    CGGCTCCTGC AAGTGCAAAA AGTGCAAATG CACCTCCTGC AAGAAGAGCT
human1r    CAGCTCCTGC AAGTGCAAAG AGTGCAAATG TACCTCCTGC AAGAAGAGCT
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human1x      CGGCTCCTGC AAATGCAAAG AGTGCAAATG CACCTCCTGC AAGAAGAGCT
human2       CGGCTCCTGC AAATGCAAAG AGTGCAAATG CACCTCCTGC AAGAAAAGCT
human3       GGACTCCTGC AAGTGCGAGG GATGCAAATG CACCTCCTGC AAGAAGAGCT
human4       AGACAACTGC AAATGCACAA CCTGCAACTG TAAAACATGT CGGAAGAGCT
icefishA     AGGATCCTGC ACTTGCACAA ACTGCTCCTG CACCAGCTGC AAGAAGAGCT
icefishB     AGGATCCTGC ACTTGCACAA ACTGCTCCTG CACCAGCTGC AAGAAGAGCT
icefishC     AGGATCCTGC ACTTGCACAA ACTGCTCTTG CACCAGTTGC AAGAAGAGCT
loach        TGCCACCTGC AAGTGTACTA ACTGCCAGTG TACGACCTGC AAGAAGAGTT
monkeya      CGACTCCTGC AAATGCAAAG AGTGCAAATG CACCTCCTGC AAGAAAAGCT
monkeyb      CGGCTCTTGC AAGTGCAAAG AGTGCAAATG CACCTCCTGC AAGAAAAGCT
mouse1       CAGCTCCTGC GCCTGCAAGA ACTGCAAGTG CACCTCCTGC AAGAAGAGCT
mouse2       TGGCGCCTGC AAATGCAAAC AATGCAAATG TACTTCCTGC AAGAAAAGCT
mouse3       GGACAAATGC AAGTGCAAGG GCTGCAAATG CACGAACTGC AAGAAGAGCT
mouse4       AGATAATTGC AAATGCACAA CCTGCAGCTG TAAAACCTGT CGTAAAAGCT
muscovy      TGGGTCCTGC AAGTGCAAGA ACTGCCGCTG CCGGAGCTGC CGCAAGAGCT
opossumB3    AGGCTCCTGC AAGTGTAAAT CCTGCCGATG TACCTCCTGC AAGAAAAGCT
opossumB1    AGGCTCCTGC AAATGTAAAT CCTGCCGCTG CACCTCCTGC AAAAAAAGCT
opossumL2    AAACTCCTGC AAATGTAAAT CTTGTCAGTG TACCTCCTGC AAGAAAAGCT
opossumL3    AGACTCTTGC AAATGCAAAT CATGCTCCTG TACCTCCTGT AAGAAAAGCT
opossumx5    AGGCTCCTGC AAATGTGCAT CCTGTCACTG TACTTCCTGC AAGAAAAGCT
perch        AGGATCCTGC ACTTGCACGA ACTGCTCCTG CACCACCTGC AAGAAGAGCT
pig          AGGCTCCTGC ACGTGCAAAG CCTGCAGATG CACCTCCTGC AAGAAGAGCT
pike         TGGATCCTGC AAGTGCTCTA ACTGCGCATG CACCAGTTGT AAGAAAAGTT
rabbit1      CAGCTCCTGC AAATGCAAAG AATGCAAATG CACCTCCTGC AAGAAGAGCT
rat1         CAGCTCCTGC GGCTGCAAGA ACTGCAAATG CACCTCCTGC AAGAAGAGCT
rat2         TGGCTCCTGC AAATGCAAAC AATGCAAATG CACCTCCTGC AAGAAAAGCT
rat3         GGACAAATGC AAATGCAAGG GCTGCAAATG CACGAACTGC AAGAAGAGCT
Ambystoma    TGGGTCATGC AAGTGTGAGA ACTGCAAGTG CACATCCTGC AAAAAAAGTT
sheep1a      TGGCTCCTGC ACCTGCAAGG CCTGCAGATG CCCCTCCTGC AAGAAGAGCT
sheep1c      TGGCTCCTGC ACCTGCAAGG CCTGCAGATG CCCCTCCTGC AAGAAGAGCT
sheep2       CGGCTCCTGC AAATGCAAAG ATTGCAAGTG CGCCTCCTGC AAGAAGAGCT
troutb       TGGATCCTGC AAGTGCTCAA ACTGCGCATG CACCAGTTGT AAGAAAAGTT
Xenopus      TACTACCTGC AGTTGCAGCA ATTGCAAGTG CACATCATGC AAGAAAAGCT

             101                                                 150
bream        GCTGCTCATG CTGCCCAGCC GGCTGCAGCA AGTGCGCCTC TGGCTGCGTG
chicken      GCTGCTCCTG CTGCCCCGCC GGCTGCAACA ACTGTGCCAA GGGCTGTGTC
cod          GTTGCGAGTG CTGTCCTTCA GGCTGCAGCA AGTGCGCCTC CGGGTGTGCG
duck         GCTGCTCCTG CTGCCCCGCC GGCTGCAACA ACTGCGCCAA GGGCTGCGTC
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echidnaA    GCTGCTCCTG CTGTCCGGTT GGGTGTGCCA AGTGTGCCCA AGGTTGTGTC
echidnaB    GCTGTTCCTG CTGTCCGGTT GGGTGTGCCA AGTGTGCCCA GGGATGCGTC
goldfish    GCTGTTCTTG TTGCCCGTCT GGTTGCAGCA AGTGCGCCTC TGGCTGCGTG
hamster1    GCTGCTCCTG CTGCCCAGTG GGCTGCTCCA AGTGTGCCCA GGGCTGCGTC
hamster2    GCTGCTCCTG CTGCCCGGTG GGCTGTGCGA AGTGCTCCCA GGGCTGCGTC
human1a     GCTGCTCCTG CTGCCCCATG AGCTGTGCCA AGTGTGCCCA GGGCTGCATA
human1b     GCTGCTCTTG CTGCCCCGTG GGCTGTGCCA AGTGTGCCCA GGGCTGTGTC
human1e     GCTGTTCCTG CTGCCCCGTG GGCTGTGCCA AGTGTGCCCA GGGCTGCGTC
human1f     GCTGCTCCTG CTGCCCCGTG GGCTGTAGCA AGTGTGCCCA AGGCTGTGTT
human1g     GCTGCTCCTG CTGCCCTGTG GGCTGTGCCA AGTGTGCCCA AGGCTGCATC
human1h     GCTGCTCCTG TTGCCCCCTG GGCTGTGCCA AGTGTGCCCA GGGCTGCATC
human1r     GCTGCTCCTG CTGCCCCATG GGCTGTGCCA AGTGTGCCCA GGGCTGCGTC
human1x     GCTGCTCCTG CTGCCCTGTC GGCTGTGCCA AGTGTGCCCA GGGCTGCATC
human2      GCTGCTCCTG CTGCCCTGTG GGCTGTGCCA AGTGTGCCCA GGGCTGCATC
human3      GCTGCTCCTG CTGCCCTGCG GAGTGTGAGA AGTGTGCCAA GGACTGTGTG
human4      GCTGTCCCTG CTGCCCCCCG GGCTGTGCCA AATGTGCCCG GGGCTGCATC
icefishA    GCTGCCCATG CTGCCCATCC GGCTGCACCA AATGCGCCTC TGGCTGCGTG
icefishB    GCTGCCCATG CTGCCCATCC GGCTGCACCA AATGCGCCTC TGGCTGCGTG
icefishC    GCTGCCCATG CTGCCCATCC GGCTGCACCA AATGCGCCTC TGGCTGTGTG
loach       GCTGTTCTTG CTGCCCCTCT GGCTGCAGTA AGTGCGCCTC TGGCTGCGTG
monkeya     GCTGCTCCTG CTGCCCCGTG GGCTGTGCCA AGTGTGCCCA GGGCTGTGTC
monkeyb     GCTGCTCCTG CTGCCCTGTG GGCTGTGCCA AGTGTGCCCA GGGCTGCATC
mouse1      GCTGCTCCTG CTGTCCCGTG GGCTGCTCCA AATGTGCCCA GGGCTGTGTC
mouse2      GCTGCTCCTG CTGCCCCGTG GGCTGTGCGA AGTGCTCCCA GGGCTGCATC
mouse3      GCTGCTCCTG CTGCCCTGCC GGATGTGAGA AGTGTGCCAA GGACTGTGTG
mouse4      GCTGTCCCTG CTGTCCCCCA GGCTGTGCCA AGTGTGCCCG GGGCTGCATC
muscovy     GCTGCTCCTG CTGCCCCGCC GGCTGCAACA ACTGCGCCAA GGGCTGCGTC
opossumB3   GCTGCTCCTG CTGCCCAGCG GGATGTGCCA AGTGTGCCCA GGGCTGTGTC
opossumB1   GCTGCTCCTG CTGCCCAGTG GGATGTGCCA AGTGTGCCCA GGGCTGTGTC
opossumL2   GCTGCTCCTG TTGCCCAGTG GGATGTGCCA AGTGTGCTCA GGGCTGTGTC
opossumL3   GCTGCTCCTG CTGCCCAGTG GGATGTGCCA AGTGTGCGCA GGGCTGTGTC
opossumx5   GCTGCTCCTG TTGCCCAGTG GGATGTGCCA AGTGTGCCCA GGGCTGTGTC
perch       GCTGCCCATG CTGCCCATCC GGCTGCCCCA AGTGCGCCTC TGGATGTGTC
pig         GCTGCTCCTG CTGCCCCGCG GGCTGTGCCA GGTGTGCCCA GGGCTGCATC
pike        GCTGCTCCTG CTGTCCTTCT GGTTGCAGCA AGTGTGCCTC AGGCTGCATA
rabbit1     GCTGCTCCTG CTGCCCCGCC GGCTGCACCA AGTGTGCCCA GGGCTGCATC
rat1        GCTGCTCCTG CTGCCCCGTG GGCTGCTCCA AATGTGCCCA GGGCTGTGTC
rat2        GCTGTTCCTG CTGCCCCGTG GGCTGTGCGA AGTGCTCCCA GGGCTGCATC
rat3        GCTGCTCCTG TTGCCCTGCA GGATGTGAGA AGTGTGCCAA GGACTGTGTT
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Ambystoma     GCTGTTCCTG CTGCCCATCG GAATGTGAGA AGTGTGGCCA GGGATGTGTT
sheep1a       GCTGCTCTTG CTGCCCTGTG GGCTGTGCCA AGTGTGCCCA GGGCTGTGTC
sheep1c       GCTGCTCATG CTGCCCCGTG GGCTGTGCCA AGTGTGCCCA GGGCTGCATC
sheep2        GCTGTTCCTG CTGCCCCGTG GGCTGTGCCA AGTGTGCCCA GGGCTGCGTC
troutb        GCTGCCCCTG CTGTCCTTCC GACTGCAGTA AATGTGCTTC AGGCTGTGTG
Xenopus       GCTGTTCCTG CTGTCCAGCT GAATGCAGCA AATGCAGCCA GGGCTGCCAC

              151                                                 200
bream         TGCAAAGGGA AGACG..... .......... ...TGCGACA CTAGCTGCTG
chicken       TGCAAGGAAC CGGCCAGC.. .......... ...AGCAAGT GCAGCTGCTG
cod           TGCAAAGACA AGACC..... .......... ...TGCGACA CTAATTGTTG
duck          TGCAAGGAGC CGGCCAGC.. .......... ...AGCAAGT GCAGCTGCTG
echidnaA      TGCAAAGAAC CCCAGACC.. .......... ...GACAAGT GCAGCTGCTG
echidnaB      TGCAAAGAGC CCCAGACC.. .......... ...GACAAGT GCAGCTGCTG
goldfish      TGTAAGGGCA ATTCC..... .......... ...TGCGGCT CCAGCTGCTG
hamster1      TGCAAAGGGG CATCG..... .......... ...GACAAGT GCACGTGCTG
hamster2      TGCAAAGAGG CTTCG..... .......... ...GACAAGT GCAGCTGCTG
human1a       TGCAAAGGGG CATCA..... .......... ...GAGAAGT GCAGCTGCTG
human1b       TGCAAAGGCT CATCA..... .......... ...GAGAAGT GCCGCTGCTG
human1e       TGCAAAGGGG CATCG..... .......... ...GAGAAGT GCAGCTGCTG
human1f       TGCAAAGGGG CGTCA..... .......... ...GAGAAGT GCAGCTGCTG
human1g       TGCAAAGGGG CATCG..... .......... ...GAGAAGT GCAGCTGCTG
human1h       TGCAAAGGGG CGTCA..... .......... ...GAGAAGT GCAGCTGCTG
human1r       TGCAAAGGGG CGTCG..... .......... ...GAGAAGT GCAGCTGCTG
human1x       TGCAAAGGGA CGTCA..... .......... ...GACAAGT GCAGCTGCTG
human2        TGCAAAGGGG CGTCG..... .......... ...GACAAGT GCAGCTGCTG
human3        TGCAAAGGCG GAGAGGCAGC TGAGGCAGAA GCAGAGAAGT GCAGCTGCTG
human4        TGCAAAGGAG GCTCA..... .......... ...GACAAGT GCAGCTGCTG
icefishA      TGCAAAGGGA AGACT..... .......... ...TGTGACA CAAGCTGCTG
icefishB      TGCAAAGGGA AGACT..... .......... ...TGTGACA CAAGCTGCTG
icefishC      TGCAAAGGGA AGACT..... .......... ...TGTGACA CAAGTTGCTG
loach         TGTAAGGGCA ATTCC..... .......... ...TGTGACT CCAGCTGTTG
monkeya       TGCAAAGGGG CGTCG..... .......... ...GAGAAGT GCAACTGTTG
monkeyb       TGCAAAGGGG CGTCG..... .......... ...GACAAGT GCAACTGCTG
mouse1        TGCAAAGGCG CCGCG..... .......... ...GACAAGT GCACGTGCTG
mouse2        TGCAAAGAGG CTTCC..... .......... ...GACAAGT GCAGCTGCTG
mouse3        TGCAAAGGTG AAGAGGGGGC CAAGGCAGAG GCCGAGAAAT GCAGCTGCTG
mouse4        TGCAAAGGGG GTTCA..... .......... ...GACAAGT GCAGCTGCTG
muscovy       TGCAAGGAGC CAGCCAGC.. .......... ...AGCAAGT GCAGCTGCTG
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opossumB3     TGCAAAGCCC CCCAGACT.. .......... ...GAGAGTT GCAGCTGCTG
opossumB1     TGCAAAGCCC CCCAGACT.. .......... ...GAGAGTT GCAGCTGCTG
opossumL2     TGCAAAGCCC CCCAGACT.. .......... ...GAGAGTT GCAGCTGTTG
opossumL3     TGCAAAGCCC CCCAGACT.. .......... ...GAGAGTT GCAGCTGTTG
opossumx5     TGCAAAGCCC CCCAGACT.. .......... ...GAGAGTT GTAGCTGCTG
perch         TGCAAAGGGA AAACT..... .......... ...TGTGACG CCGCCTGCTG
pig           TGCAAAGGGG CCTCG..... .......... ...GACAAGT GCAGCTGCTG
pike          TGCAAGGGCA AGACC..... .......... ...TGTGATA CCAGCTGCTG
rabbit1       TGCAAAGGGG CATCG..... .......... ...GACAAGT GCAGCTGCTG
rat1          TGCAAAGGTG CCTCG..... .......... ...GACAAGT GCACGTGCTG
rat2          TGCAAAGAGG CTTCG..... .......... ...GACAAGT GCAGCTGCTG
rat3          TGCAAAGGCG AAGAG..... .GGGGCCAAG GCCGAGAAAT GCAGCTGCTG
Ambystoma     TGCAAAGGAG GGTCA..... .......... TCCGAGAAAT GCAGCTGTTG
sheep1a       TGCAAAGGGG CCTCG..... .......... ...GACAAGT GCAGCTGCTG
sheep1c       TGCAAAGGGG CCTCG..... .......... ...GACAAGT GCAGCTGCTG
sheep2        TGCAAAGGGG CCTCG..... .......... ...GACAAGT GCAGCTGCTG
troutb        TGCAAGGGCA AGACA..... .......... ...TGCGATA CCAGCTGTTG
Xenopus       TGTGAAAAGG GAAGC..... .......... ...AAGAAGT GCAGCTGCTG

                    201
bream         CCAGTGA
chicken       CCACTGA
cod           CCAGTGA
duck          CCACTGA
echidnaA      CCAGTGA
echidnaB      CCAGTGA
goldfish      TCAATGA
hamster1      TGCCTAA
hamster2      CGCCTGA
human1a       TGCCTGA
human1b       TGCCTGA
human1e       TGCCTGA
human1f       CGACTGA
human1g       CGCCTGA
human1h       TGCCTGA
human1r       TGCCTGA
human1x       TGCCTGA
human2        CGCCTGA
human3        CCAGTGA
```

human4	CCCATGA
icefishA	TCAGTGA
icefishB	TCAGTGA
icefishC	TCAGTGA
loach	TCAATGA
monkeya	TGCCTGA
monkeyb	CGCCTGA
mouse1	TGCCTGA
mouse2	TGCCTGA
mouse3	CCAGTGA
mouse4	TCCCTGA
muscovy	CCACTGA
opossumB3	CCACTGA
opossumB1	CCAGTGA
opossumL2	CCACTGA
opossumL3	CCACTGA
opossumx5	TCAGTGA
perch	CCAGTGA
pig	TGCCTGA
pike	TCAGTGA
rabbit1	CGCCTGA
rat1	TGCCTGA
rat2	CGCCTGA
rat3	CCAGTGA
Ambystoma	CAACTAA
sheep1a	CGCCTGA
sheep1c	TGCCTGA
sheep2	TGCCTGA
troutb	TCAGTGA
Xenopus	TAACTGA

Appendix II - Complete MT Protein Data Set

59 species 72 characters
All sequences are taken from Kagi (1993), unless specified with a footnote.
Gaps introduced into sequences to permit alignment are represented "-" .

```
pike        MDP--CECSKTG-SCNCGGSCKCSNCACT--SCKK-SCCSCCPSGCSKCASGCICKG------KTCDTSCCQ
human1a     MDP---NCSCAT-GGSCTCTGSCKCKECKCNSCKK-SCCSCCPMSCAKCAQGCICKG------ASEKCSCCA
human1b     MDP---NCSCTT-GGSCACAGSCKCKECKCTSCKK-CCCSCCPVGCAKCAQGCVCKG------SSEKCRCCA
human1e     MDP---NCSCAT-GGSCTCAGSCKCKECKCTSCKK-SCCSCCPVGCAKCAQGCVCKG------ASEKCSCCA
human1f     MDP---NCSCAA-GVSCTCAGSCKCKECKCTSCKK-SCCSCCPVGCSKCAQGCVCKG------ASEKCSCCD
human1g     MDP---NCSCAA-GVSCTCASSCKCKECKCTSCKK-SCCSCCPVGCAKCAQGCICKG------ASEKCSCCA
human1h     MDP---NCSCEA-GGSCACAGSCKCKKCKCTSCKK-SCCSCCPLGCAKCAQGCICKG------ASEKCSCCA
human1i     MDP---NCSCAA-GVSCTCAGSCKCKECKCTSCKK-SCCSCCPVGCAKCAQGCICKG------ASEKCSCCA
human1k     MDP---NCSCAAAGVSCTCASSCKCKECKCTSCKK-SCCSCCPVGCAKCAQGCICKG------ASEKCSCCA
human11     MDP---NCSCSP-VGSCACAGSCKCKECKCTSCKK-SCCSCCPVGCAKCAQGCICKG------TSDKCSCCA
bovine1a    MDP---NCSCPT-GGSCSCAGSCTCKACRCPSCKK-SCCSCCPVGCAKCAQGCVCKG------ASDKCSCCA
rat1        MDP---NCSCST-GGSCTCSSSCGCKNCKCTSCKK-SCCSCCPVGCSKCAQGCVCKG------ASDKCTCCA
monkey1     MDP---NCSCAT-GVSCTCADSCKCKECKCTSCKK-SCCSCCPVGCAKCAQGCVCKG------ASEKCNCCA
*chamster1  MDP---NCSCST-GSTCTCSSSCGCKDCKCTSCKK-SCCSCCPVGCSKCAQGCVCKG------ASDKCTCCA
mouse1      MDP---NCSCST-GGSCTCTSSCACKNCKCTSCKK-SCCSCCPVGCSKCAQGCVCKG------AADKCTCCA
rabbit1a    MDP---NCSCAT-GNSCTCASSCKCKECKCTSCKK-SCCSCCPAGCTKCAQGCICKG------ASDKCSCCA
horse1a     MDP---NCSCPT-GGSCTCAGSCKCKECRCTSCKK-SCCSCCPGGCARCAQGCVCKG------ASDKCSCCA
horse1b     MDP---NCSCVA-GESCTCAGSCKCKQCRCASCKK-SCCSCCPVGCAKCAQGCVCKG------ASDKCSCCA
pig1        MDP---NCSCPT-GGSCSCAGSCTCKACRCTSCKK-SCCSCCPAGCARCAQGCICKG------ASDKCSCCA
sheep1b     MDP---NCSCPT-SGSCSCAGSCTCKACRCPSCKK-SCCSCCPVGCAKCAQGCVCKG------ASDKCSCCA
sheep1c     MDP---NCSCST-GSSCSCAGSCTCKACRCPSCKK-SCCSCCPVGCAKCAQGCICKG------ASDKCSCCA
possumB1    MDP---KCNCES-GGSCTCAGSCKCKSCRCTSCKK-SCCSCCPVGCAKCAQGCVCKAP-----QTESCSCCQ
possumL3    MDP---NCNCAN-GGSCTCGDSCKCKSCSCTSCKK-SCCSCCPVGCAKCAQGCVCKAP-----QTESCSCCH
possumL2    MDP---DCDCVS-GGSCTCANSCKCKSCQCTSCKK-SCCSCCPVGCAKCAQGCVCKAP-----QTESCSCCH
possumB3    MDP---NCDCVS-GSSCTCAGSCKCKSCRCTSCKK-SCCSCCPAGCAKCAQGCVCKAP-----QTESCSCCH
possumx5    MDP---NCDCGN-GASCTCAGSCKCASCHCTSCKK-SCCSCCPVGCAKCAQGCVCKAP-----QTESCSCCQ
echidnaA    MDP--QDCGCAT-GGSCACATSCKCKDCRCTSCKK-SCCSCCPVGCAKCAQGCVCKEP-----QTDKCSCCQ
echidnaB    MDP--QNCGCAT-GGSCTCAGSCKCKDCRCTSCKK-SCCSCCPVGCAKCAQGCVCKEP-----QTDKCSCCQ
human2      MDP---NCSCAA-GDSCTCAGSCKCKECKCTSCKK-SCCSCCPVGCAKCAQGCICKG------ASDKCSCCA
mouse2      MDP---NCSCAS-DGSCSCAGACKCKQCKCTSCKK-SCCSCCPVGCAKCSQGCICKE------ASDKCSCCA
rat2        MDP---NCSCAT-DGSCSCAGSCKCKQCKCTSCKK-SCCSCCPVGCAKCSQGCICKE------ASDKCSCCA
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   *chamster2MDP---NCSCAT-DGSCSCAGSCKCKECKCTTCKK-SCCSCCPVGCAKCSQGCVCKE------ASDKCSCCA
1. rabbit2a   MDP---NCSCAAAGDSCTCANSCTCKACKCTSCKK-SCCSCCPPGCAKCAQGCICKG------ASDKCSCCA
   rabbit2b   MDP---NCSCAT-GDSCTCASSCKCKECKCTSCKK-SCCSCCPAGCTKCAQGCICKG------ASDKCSCCA
   rabbit2c   MDP---NCSCATAGDSCTCANSCTCKACKCTSCKK-SCCSCCPPGCAKCAQGCICKG------ASDKCSCCA
   rabbit2d   MDP---NCSCAT-RDSCACASSCKCKECKCTSCKK-SCCSCCPAGCTKCAQGCICKG------ASDKCSCCA
   rabbit2e   MDP---NCSCAT-RDSCACASSCKCKECKCTSCKK-SCCSCCPAGCTKCAQGCICKG------ALDKCSCCA
   bovine2    MDP---NCSCTA-GESCTCAGSCKCKDCKCASCKK-SCCSCCPVGCAKCAQGCVCKG------ASDKCSCCA
   monkey2    MDP---NCSCVA-GDSCTCAGSCKCKECKCTSCKK-SCCSCCPVGCAKCAQGCICKG------ASDKCNCCA
2. dog        MDP---DCSCST-GGSCTCAGSCKCKECKCTSCKK-SCCSCCPVGCAKCAQGCICKG------ASDKCSCCA
   dolphin2   MDP---NCSCTA-GGSCACPGSCKCKECKCTSCKK-SCCSCCPVGCAKCAQGCICKG------ASDKCSCCA
   human3     MDP--ETCPCPS-GGSCTCADSCKCEGCKCTSCKK-SCCSCCPAECEKCAKDCVCKGGEAAEAEAEKCSCCQ
3. bovine3    MDP--ETCPCPT-GGSCTCSDPCKCEGCTCASCKK-SCCSCCPAECEKCAKDCVCKGGEGAEAEEKKCSCCQ
4. horse3     MDP--ETCPCPT-GGSCTCSGECKCEGCKCTSCKK-SCCSCCPAECEKCAKDCVCKGGEGAEAEAEKCSCCQ
   mouse3     MDP--ETCPCPT-GGSCTCSDKCKCKGCKCTNCKK-SCCSCCPAGCEKCAKDCVCKGEEGAKAEAEKCSCCQ
   rat3       MDP--ETCPCPT-GGSCTCSDKCKCKGCKCTNCKK-SCCSCCPAGCEKCAKDCVCKGEEGAK--AEKCSCCQ
5. sheep3     MDP--EACPCPT-GGSCTCSDSCKCEGCTCASSKK----SCCPAECEKCAKDCVCKGGEGAEAEEKKCGCCQ
   mouse4     MDP--GECTCMS-GGICICGDNCKCTTCSCKTCRK-SCCPCCPPGCAKCARGCICKG------GSDKCSCCP
6. human4     MDP--RECVCMS-GGICMCGDNCKCTTCNCKTCRK-SCCPCCPPGCAKCARGCICKG------GSDKCSCCP
   chicken    MDP--QDCTCAA-GDSCSCAGSCKCKNCRCRSCRK-SCCSCCPAGCNNCAKGCVCKEP-----ASSKCSCCH
   pigeon1    MDS--QDCPCAA-GGTCTCGDNCKCKNCKCTSCKK-GCCSCCPAGCAKCAQGCVCKGP-----PSAKCSCCK
   pigeon2    MDP--QDCTCAA-GDSCSCAGSCKCKNCRCQSCRK-SCCSCCRASCSNCAKGCVCKEP-----SSSKCSCCH
   loach      MDP--CDCSKTG-TCNCGATCKCTNCQCT--TCKK-SCCSCCPSGCSKCASGCVCKG------NSCDSSCCQ
   plaice     MDP--CECSKTG-TCNCGGSCTCKNCSCT--TCNK-SCCPCCPSGCPKCASGCVCKG------KTCDTSCCQ
   troutB     MDP--CECSKTG-SCNCGGSCKCSNCACT--SCKK-SCCPCCPSDCSKCASGCVCKG------KTCDTSCCQ
   troutA     MDP--CECSKTG-SCNCGGSCKCSNCACT--SCKKASCCDCCPSGCSKCASGCVCKG------KTCDTSCCQ
   Xenopus    MDPQDCKCETGA-SCSCGTTCSCSNCKCT--SCKK-SCCSCCPAECSKCSQGCHCEK------GSKKCSCCN
7. Ambystoma MD---CACATGG-SCSCAGSCKCENCKCT--SCKK-SCCSCCPSECEKCGQGCVCKGG-----SSEKCSCCN
```

1. ghamster = golden hamster, translated from Duguid *et al.*, 1988
2. Kobayashi *et al.*, 1997.
3. Poutney *et al.*, 1994.
4. Poutney et al., 1994.
5. Holloway *et al.*, 1996.
6. Quaife *et al.*, 1994.
7. Saint-Jacques *et al.*, 1998.

* Chinese hamster

ADDENDUM

Page vi

Abbreviations:
MT - metallothionein
The isoforms of metallothionein are designated MT1, MT2, MT3 and MT4; the subforms of isoforms are designated MT1a, MT1b etc.
The genes for metallothionein isoforms are designated *MT1*, *MT2*, *MT3* and *MT4*; *MT1a*, *MT1b* etc.

ERRATA

Page vii, line 17	Replace "-4-chloro-3-inoyl-β-" with "-4-chloro-3-indolyl-β-".
Page x, line 21	Replace "metallotionein" with "metallothionein".
Page 4, line 21	Replace "arefact" with "artefact".
Page 5, line 4	Replace "metallothinein" with "metallothionein".
Page 7, line 15	Replace "half-life for than" with "half-life than".
Page 12, line 4	Replace "ehance" with "enhance".
Page 13, line 16	Replace "enhance" with "enhanced".
Page 15, line 7	Replace "tranfected" with "transfected".
Page 16, lines 30,31	Replace "siezure" with "seizure".
Page 17, line 13	Replace "resistent" with "resistant".
Page 22, line 27	Replace "contibution" with "contribution".
Page 22, line 29	Replace "tht" with "that".
Page 23, line 4	Replace "marsupuial" with "marsupial".
Page 27, line 7	Replace "coolling" with "cooling".
Page 29, line 6	Replace "coolled" with "cooled".
Page 31, line 13	Replace "innoculated" with "inoculated".
Page 31, line 15	Replace "pre-coolled" with "pre-cooled".
Page 34, line 9 Page 44, line 13	Replace "coolling" with "cooling".
Fig. 2.2, legend lines 2, 6	Replace "Stratgene" with "Stratagene".
Page 44, line 14	Replace "fractionation" with "fractionation.".
Page 49, line 14	Replace "for1 hour" with "for 1 hour".

Page 49, line 25	Replace "spoolled" with "spooled".
Page 71, line 14	Replace "membrane-bound RNA from various opossum tissues" with "membrane-bound RNA from various opossum tissues (liver, kidney, spleen, heart, lung, muscle, brain, tongue, salivary gland and testis)".
Page 52, line 24	Replace "ployacrylamide" with "polyacrylamide".
Page 72, line 18	Replace "metallothioniens" with "metallothioneins".
Page 73, line 3	Replace "metallothineins" with "metallothioneins".
Page 74, line 19	Replace "*Tachgyglossus*" with "*Tachyglossus*".
Page 88, line 17	Replace "(Nelson, 1988)" with "(Nelson, 1988).".
Page 88, line 28 Page 89, line 20	Replace " adult opossums" with "adult (12 months) male opossums".
Fig. 4.6, legend line 1	Replace "Levels of MT mRNA" with "Relative levels of MT mRNA".
Fig. 4.15, legend line 15	Replace "inthe" with "in the".
Page 97, line 8	Replace "tissues types" with "tissue types".
Page 97, line 11	Replace "sheep has been" with "sheep have been".
Page 97, line 14	Replace "eutheria" with "eutherian".
Page 99, line 2	Replace "1977)" with "1977).".
Page 121, line 30	Replace "dataset" with "data set".
Page 124, line 12	Replace "sections" with "section".
Page 128, line 8	Replace "similarto" with "similar to".
Page 134, line 28	Replace "errorr" with "error".
Page 139, line 22	Replace "milion" with "million".
Page 142, line 9	Replace "engineeering" with "engineering".
Page 146, line 17	Replace "independently" with "independently.".
Page 151, line 7	Replace "Carpenter, 1993)" with "Carpenter, 1993).".
Page 156, line 7	Replace "Inforation" with "Information".
Page 160, line 28	Replace "&Seguin" with "& Seguin".
Page 164, line 24	Replace "inhibtory" with "inhibitory".
Page 166, line 5	Replace "Maximuum" with "maximum".